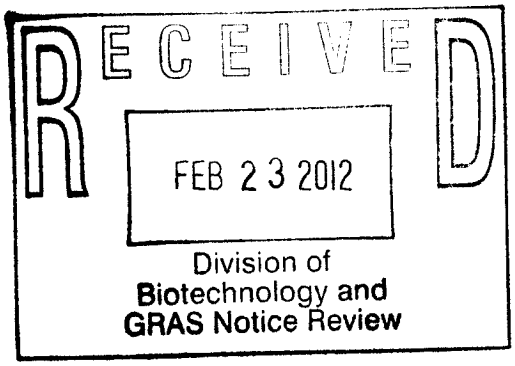
# Spherix Consulting, Inc.

February 22, 2012




Office of Food Additive Safety
HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

To Whom It May Concern:

Enclosed please find three copies of "Generally Recognized As Safe (GRAS) Notification for Cow's Milk-Derived Lactoferrin as a Component of Cow's Milk-Based Infant Formulas, Cow's Milk Products, and Chewing Gum". This GRAS notification has been prepared by Spherix Consulting, Inc. for Morinaga Milk Industry Co., Ltd..

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, Ph.D., D.A.B.T., CEO, Spherix Consulting, Inc., 6430 Rockledge Drive, Suite 503, Bethesda, Maryland, 20817, Telephone: 301-897-0611; Facsimile: 301-897-2567; Email: ckruger@spherix.com.

Should you have any questions or concerns, please contact me at the number listed above.

Sincerely,

(b) (6)

Claire L, Kruger, Ph.D., D.A.B.T.
Chief Executive Officer

Enclosures:

Three copies of "Generally Recognized As Safe (GRAS) Notification for Cow's Milk-Derived Lactoferrin as a Component of Cow's Milk-Based Infant Formulas, Cow's Milk Products, and Chewing Gum"

Three copies of the GRAS Panel Consensus Statement for the above-referenced GRAS Notification

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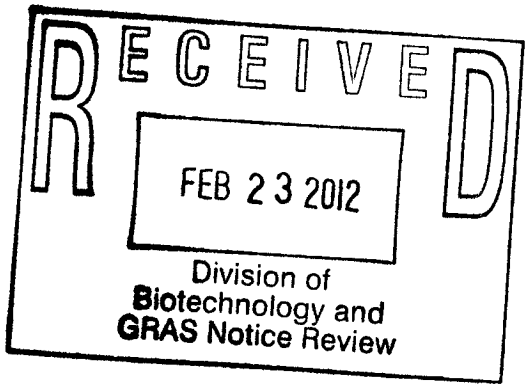
# GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR COW'S MILK-DERIVED LACTOFERRIN AS A COMPONENT OF COW'S MILK-BASED INFANT FORMULAS, COW'S MILK PRODUCTS, AND CHEWING GUM




**Prepared for:**

Morinaga Milk Industry Co., Ltd.
33-1, Shiba 5-Chome
Minato-ku, Tokyo 108-8384
Japan

**Prepared by:**

Spherix Consulting, Inc.
6430 Rockledge Drive #503
Bethesda, MD 20817
USA

June 22, 2011

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**TABLES**



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# I. GRAS EXEMPTION CLAIM

## A. NAME AND ADDRESS OF THE SPONSOR

### 1. BUSINESS ADDRESS

Morinaga Milk Industry Co., Ltd.
33-1, Shiba 5-Chome, Minato-ku
Tokyo 108-8384, Japan
Tel: 81-3-3798-0152
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### 2. MAILING ADDRESS

interntl@morinagamilk.co.jp

## B. COMMON OR USUAL NAME OF GRAS SUBSTANCE

The substance that is the subject of this Generally Recognized As Safe (GRAS) determination is cow's milk-derived lactoferrin (cMDLf).

## C. INTENDED USE AND CONSUMER EXPOSURE

Morinaga Milk Industry Co., Ltd. intends to supplement cow's milk-based infant formulas and cow's milk-based products with cMDLf. The intended uses are in cow's milk-based infant formulas [powdered (100 mg/100 g), liquid concentrates (26 mg/100 ml), and ready-to-feed formulas (13 mg/100 ml)], yogurt (100 mg/100 g), powdered milk (400 mg/100 g), ice cream and sherbets (200 mg/ 100 g), and chewing gum (30 mg/g).

## D. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of cow's milk-derived lactoferrin (cMDLf) in cow's milk-based infant formulas and cow's milk-based products in yogurt (100 mg/100 g), powder milk (400 mg/100 g), infant formula (powdered (100 mg/100 ml), liquid concentrates (26 mg/100 ml) and ready to feed formulas (13 mg/100 ml), milk desserts (200 mg/ 100 g), and chewing gum (30 mg/g) is based upon scientific procedures as described under 21 CFR §170.30(b).

The intake of cMDLf from the specified intended uses has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), Section 201(s). To demonstrate that cMDLf is safe, and GRAS, under the intended conditions of use, the safety of the intake of cMDLf has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

The proposed use of cMDLf to supplement levels of this protein in selected cow's milk-based food products has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. In the United States, cMDLf has been determined safe and GRAS for use as an ingredient in sport and functional foods at concentrations of 100 mg/serving (GRN 77). cMDLf has also been determined safe and GRAS for use as a component of an antimicrobial spray for application to uncooked beef to prevent microbial contamination under GRN 67 and GRN 130.

2. cMDLf has a long and safe history of ingestion. Milk from cows has been consumed by the human population for centuries. Milk protein is a combination of caseins and whey proteins. Caseins account for 79.2% (27 mg/ml milk) of the total milk proteins. The remaining 20.8% (7.1 mg/ml milk) is whey protein. cMDLf, which is a whey protein, accounts for 0.3% (0.1 mg/ml milk) of the total protein or 1.4% of whey protein in milk.

3. cMDLf manufactured for Morinaga Milk Industry Co., Ltd. complies with established food grade specifications and utilizes only food grade raw materials and processing aids. The product consists of 4.2% moisture, 1.3 % ash, and 94.5% protein, of which ≥ 96% is lactoferrin. The current production facilities comply with either the requirements of International Food Standard Version 5 or ISO 9001:2008 and ISO 14001:2004.

4. Infant formulas with added cMDLf are approved for use and sold in Japan, Taiwan, Pakistan, Indonesia, and China by Morinaga Milk Industry Co., Ltd. In Japan, the formulas have been certified by the Japanese Ministry of Health, Labor, and Welfare as Special Nutritious Foods according to the Nutrition Improvement Law. Morinaga's cMDLf has been listed on the "natural additive list" in Japan since 1989 and was added to the "existing additive list" in Japan in 1995. In Japan, there is no specific restriction

for cMDLf because it is considered a natural material. The proposed GRAS level of cMDLf (100 mg/100 g powdered infant formula) to be added to cow-milk based infant formula is similar to the level of cMDLf in currently marketed and consumed Morinaga Milk infant formula products (80 mg/100 g powdered infant formula).

5. Since the release of Morinaga's infant formula products in 1986 and 1989, Morinaga has sold annually approximately 3,200 metric tons (76,800 metric tons total) and 3,500 metric tons (73,500 metric tons total) in Japan for the 0- to 9-month formulation and 9-month to 3-yr formulation, respectively. With consumption of these formulas with added cMDLf by over a million infants and toddlers in Japan since 1986, there have been no reported significant health problems, including allergenic reactions or autoimmunity attributable to cMDLf, associated with either of these two products containing cMDLf based on post-marketing surveillance.

6. The mean EDIs of cMDLf from addition to infant formulas are 100.3 mg/day for infants 0 to 4 mo and 87.4 mg/day for infants 5 to 11 mo. The 90th percentile intakes for these age groups are 145.5 mg/day and 129 mg/day, respectively. After cMDLf addition to infant formulas, the cMDLf exposure to this protein for infants in these age categories is approximately two-times the background exposure from unsupplemented cow-milk based infant formula.

7. The mean and 90th percentile EDIs by the total population of cMDLf from addition to proposed products excluding infant formulas are 142 mg/day and 273 mg/day (2.7 mg/kg/day and 5.8 mg/kg/day, respectively). Similarly, for all other population groups, the exposure to cMDLf from addition to the proposed products increases exposure by two- to three-times background. (Non-milk products (chewing gum) containing cMDLF will be appropriately labeled as containing cow's milk proteins.)

8. The EDIs for cMDLf have been concluded to be safe and GRAS based on ADME studies, animal toxicology studies, studies evaluating physiologic effects, and clinical studies in adults, children, and infants. These published studies support the safety of intake of cMDLf at the proposed levels.

9. Digestion and metabolic fate of lactoferrin has been evaluated from studies of both human milk-derived and cow's milk-derived lactoferrin. Lactoferrin from both sources is handled similarly by the body. Lactoferrin that is absorbed from the gastrointestinal tract partitions into the lymph and then appears in the blood. Lactoferrin is rapidly removed from the systemic circulation by distribution into the spleen, liver, and

kidneys while the iron portion is transferred to the liver for its transport into the bone marrow. Intact lactoferrin is detected in the feces and urine of infants.

10. While very few patients with cow's milk allergy (CMA) have antibodies to bovine lactoferrin, no evidence exists that cMDLf is a clinically relevant allergen.

11. It has been shown that infants fed with formula containing added cMDLf developed anti-cMDLf antibodies. Exclusively breast-fed infants develop anti-human lactoferrin antibodies, and autoimmune adults have antibodies that recognize both human and cMDLf. However, there is no evidence that cMDLf causes, promotes, exacerbates, or resolves autoimmunity in infants or adults.

12. The safety of cMDLf produced by Morinaga Milk Industry Co., Ltd. was evaluated in an acute toxicity study, a four-wk oral toxicity study, a thirteen-wk oral toxicity study, a chronic oral toxicity study and genotoxicity assays. cMDLf is not acutely toxic or genotoxic. cMDLf administered by oral intubation to rats for 13 wks did not result in toxicologically significant treatment-related changes. Thus, under the conditions of this study, the no-observed-adverse-effect level (NOAEL) of cMDLf was estimated to be in excess of 2,000 mg/kg/day. The chronic toxicity study was not available as a full report and therefore was not used to derive a NOAEL.

13. Numerous published studies on the effects of oral ingestion of cMDLf in adults, children, and infants corroborate the GRAS status of cMDLf for its proposed uses. Of the 37 studies in humans, 17 have been conducted using cMDLf supplied by Morinaga Milk Industry. cMDLf is safe and well tolerated under the conditions of administration in the clinical studies at doses that range in adults and children from 100 mg/day to 3.6 g/day and with durations ranging from one wk to one yr. Studies carried out in both healthy and health-compromised adults and children reported no treatment related side effects attributed to oral ingestion of cMDLf. Administration of cMDLf at 200 mg/day for up to 90 d in pregnant women was well tolerated (highest dose tested in this target population). In term and preterm infants, exposure to cMDLf from infant formulas has been studied using concentrations ranging from 10 mg/100 mL (0.01%) to 285 mg/100 mL (0.285%), comprising durations ranging from two wks to one yr. Resulting intake of cMDLf is up to 150 mg/kg/day. No treatment related adverse effects have been reported in infants.

Determination of the GRAS status of cMDLf supplied by Morinaga Milk Industry under the intended conditions of use has been made through the deliberations of Dr. Stephen Taylor, Dr. Lloyd Mayer, Dr. A. Wallace Hayes, and Dr. Roger Clemens. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of cMDLf and the potential human exposure to cMDLf resulting from its intended use in milk-derived products, chewing gum and cow's milk-based infant formulas and have concluded:

*There is no evidence in the available information on cMDLf that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when cMDLf is used at levels that might reasonably be expected from the proposed applications. cMDLf is GRAS for use in foods as proposed by Morinaga Milk Industry Co., Ltd.*

Therefore, cMDLf is safe and GRAS at the proposed levels of addition to foods. cMDLf is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

## E. AVAILABILITY OF INFORMATION

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, Ph.D., D.A.B.T., CEO, Spherix, Incorporated, 6430 Rockledge Drive, Westmoreland Bldg, Suite 503, Bethesda, Maryland, 20817, Telephone: 301-897-0611; Facsimile: 301-897-2567; Email: ckruger@spherix.com.

## F. SIGNATURE

Pursuant to the criteria provided in proposed 21 CFR 170.36, Morinaga Milk Industry Co., Ltd. hereby notifies the Food and Drug Administration that the use of cMDLf in foods under the intended conditions of use is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because Morinaga Milk Industry Co., Ltd. has determined that such use is Generally Recognized As Safe through scientific procedures.

(b) (6)

Signature

February 22, 2012
Date

Claire L. Kruger, CEO, Spherix Consulting, Inc.
Authorized Representative of Morinaga Milk
Industry Co., Ltd.

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# II. DESCRIPTION OF SUBSTANCE

## A. PHYSICAL AND CHEMICAL COMPOSITION

As cited in GRN 42, cow lactoferrin (cLf) is a 689 amino acid glycoprotein with a molecular weight of approximately 75 to 80-kDa (Mead and Tweedie, 1990; Pierce et al., 1991, and Table 1). The mature protein contains intramolecular disulfide bonds, is absent of free sulfhydryl groups, and folded into two symmetrical lobes (Spik et al., 1988; van Halbeek et al., 1981). Each lobe binds a metal atom in synergy with the carbonate ion ($CO_3^{2-}$) (Figure 1). Lactoferrin primarily binds $Fe^{2+}$ or $Fe^{3+}$, but can also bind to $Cu^{2+}$, $Zn^{2+}$ and $Mn^{2+}$ (van der Strate et al., 2001). Because of its ability to bind $Fe^{3+}$ reversibly, cLf can exist in iron ($Fe^{3+}$)-free (apo-cLf) or iron-saturated (holo-cLf) states (Drago, 2006) and its three-dimensional conformation depends on whether or not it is bound to iron (Schanbacher et al., 1993). Apo-cLf has an open conformation, while holo-cLf has a closed conformation (Yeşim and Özgüneş, 2005). Holo-cLf is more resistant to proteolysis and thermal denaturation (Yeşim and Özgüneş, 2005; Paulsson et al., 1993). Cow milk-derived lactoferrin (cMDLf) contains four N-linked glycans, which are composed of different amounts of N-acetyllactosaminic acid, galactose, mannose, fucose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid residues (Coddeville et al., 1992; Spik et al., 1994). The isoelectric point (pI) of cMDLf is 8.2 to 8.9 by chromatofocusing (Shimazaki et al., 1993) and 9.5 to 10 by isoelectric focusing (Yoshida, 1991). Apo-cMDLf has an absorbance of 12.7 when measured at 280 nm whereas the absorbance of holo-cMDLf is 0.460 at 470 nm (Aisen and Leibman, 1972).

| Table 1. Physical and Chemical Properties of Cow's Milk-Derived Lactoferrin | | |
|---|---|---|
| **Property** | Values | **Reference** |
| Molecular mass (Da) | | |
| *Sedimentation co-efficient* | 77,100 ± 1,500 | (Castellino et al., 1970) |
| *SDS-PAGE* | 76,000 ± 2,400 | (Castellino et al., 1970) |
| *Iron titration* | 78,500 | (Aisen and Leibman, 1972) |
| Isoelectric point (pH) | | |
| *Chromatofocusing* | 8.2-8.9 | (Shimazaki et al., 1993) |
| *Isoelectric focusing* | 9.5-10.0 | (Yoshida, 1991) |
| Absorption spectra | | (Aisen and Leibman, 1972) |
| *Apo-form at 280 nm* | 12.7 | |
| *Holo-form at 470 nm* | 0.460 | |
| Iron-binding | | (Aisen and Leibman, 1972) |
| *Equilibrium dialysis ($K_1$ x $10^{-4}$)* | 3.73 | |
| Thermal denaturation | | (Paulsson et al., 1993) |
| *Apo-Lf denaturation ($T_{max}$: °C)* | 71 ± 0.3 and 90 ± 0.3 | |
| *Apo-Lf enthalpy ($\Delta H_{cal}$: J/g)* | 12 ± 0.4 and 2 ± 0.5 | |
| *Holo-Lf denaturation ($T_{max}$: °C)* | 65 ± 0.3 and 93 ± 0.3 | |
| *Holo-Lf enthalpy ($\Delta H_{cal}$: J/g)* | 2 ± 1.2 and 37 ± 1.3 | |

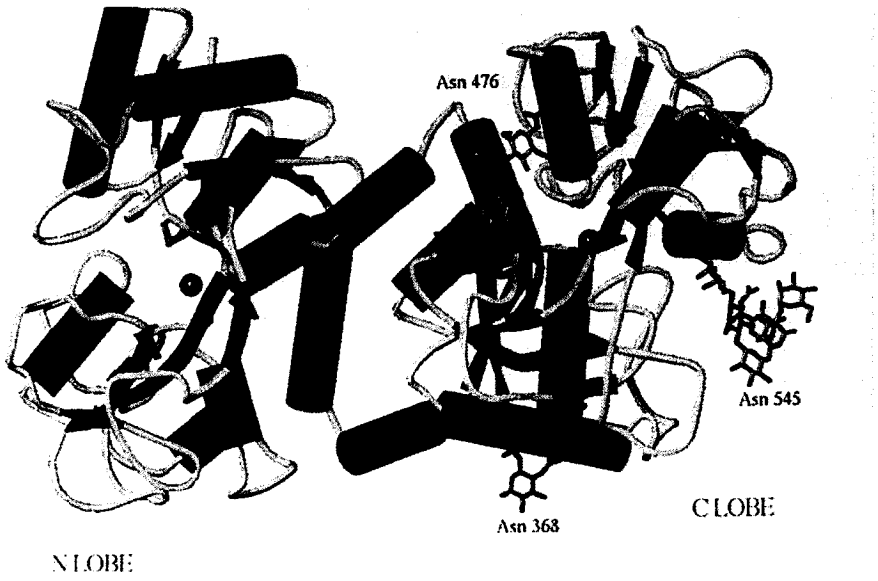


**Figure 1: Schematic of diferric Cow Lactoferrin at a Resolution of 2.8 Angstroms**
(Moore et al., 1997)

## B. MANUFACTURING PROCESS

### 1. GENERAL DESCRIPTION OF THE PRODUCTION PROCESS

cMDLf is extracted either from sweet whey (cMDLf-1), a cheese production byproduct, or directly from skim milk (cMDLf-2). When sweet whey is the starting material, the entire production process is carried out at Milei GmbH located at Kemptener Strasse 91, 88299 Leutkirch, Germany. Milei GmbH has been certified for the development, production, and sales of products from whey and milk by the certifying body TUV SUD Management Service GmbH (Certificate registration number: 12 100/104 21695 TMS). When skim milk is used as the starting material, crude cMDLf is prepared at the Riedlingen plant of Allgäuland-Käsereien GmbH located at 88499 Riedlingen, Göffinger Straße 6, Germany. Crude cMDLf is then transported to Milei GmbH for further processing. The Riedlingen plant of Allgäuland-Käsereien GmbH has been certified for processing, manufacturing, and sales of butter, cream cheese preparations, farmer's cheese (quark), yogurt, UHT milk, UHT cream and milk protein concentrate from dairy products as required by the International Food Standard, Version 5, August 2007 (Certificate registration number: F12683-2010-01) by the certifying body LACON GMBH on November 10, 2010.

The production process is summarized in Figure 2. Skim milk (unpasteurized) or sweet whey is cooled to below 8°C to prevent microbial growth (Step A), prefiltered with a 5 μM cloth (Step B1), and microfiltered with a 1 μM cloth (Step B2) to remove the insoluble material, contaminating microbes, and fat. The filtrate is bound to an ion exchange column containing CM Sephadex C-50 (currently used) or another food-grade resin, such as SP Sepharose Big Beads (proposed) (Step C), rinsed thoroughly with demineralized water, and washed with a 1.6 % NaCl solution to remove contaminants weakly bound to the resin. cMDLf is then eluted with a 10% NaCl solution and desalted by ultrafiltration using Tangential Flow Filtration (Steps D). Importantly, the ion exchange columns are designed to prevent carry-over of the resins into the cMDLf eluate and the ultrafiltration step removes the residual low molecular weight contaminants eluted with cMDLf. The pH of the cMDLf ultrafiltrate is then adjusted to approximately 5.8, which increases the stability of cMDLf during sterilization, and sterilized (Step E). The sterilized cMDLf solution is further concentrated with a second ultrafiltration step (Step F). The concentrated cMDLf is then freeze-dried (Step G), and pulverized to desired mesh size (Step H) before packaging (Step I). Critical control points and appropriate procedures to correct out-of-specification issues are in place.

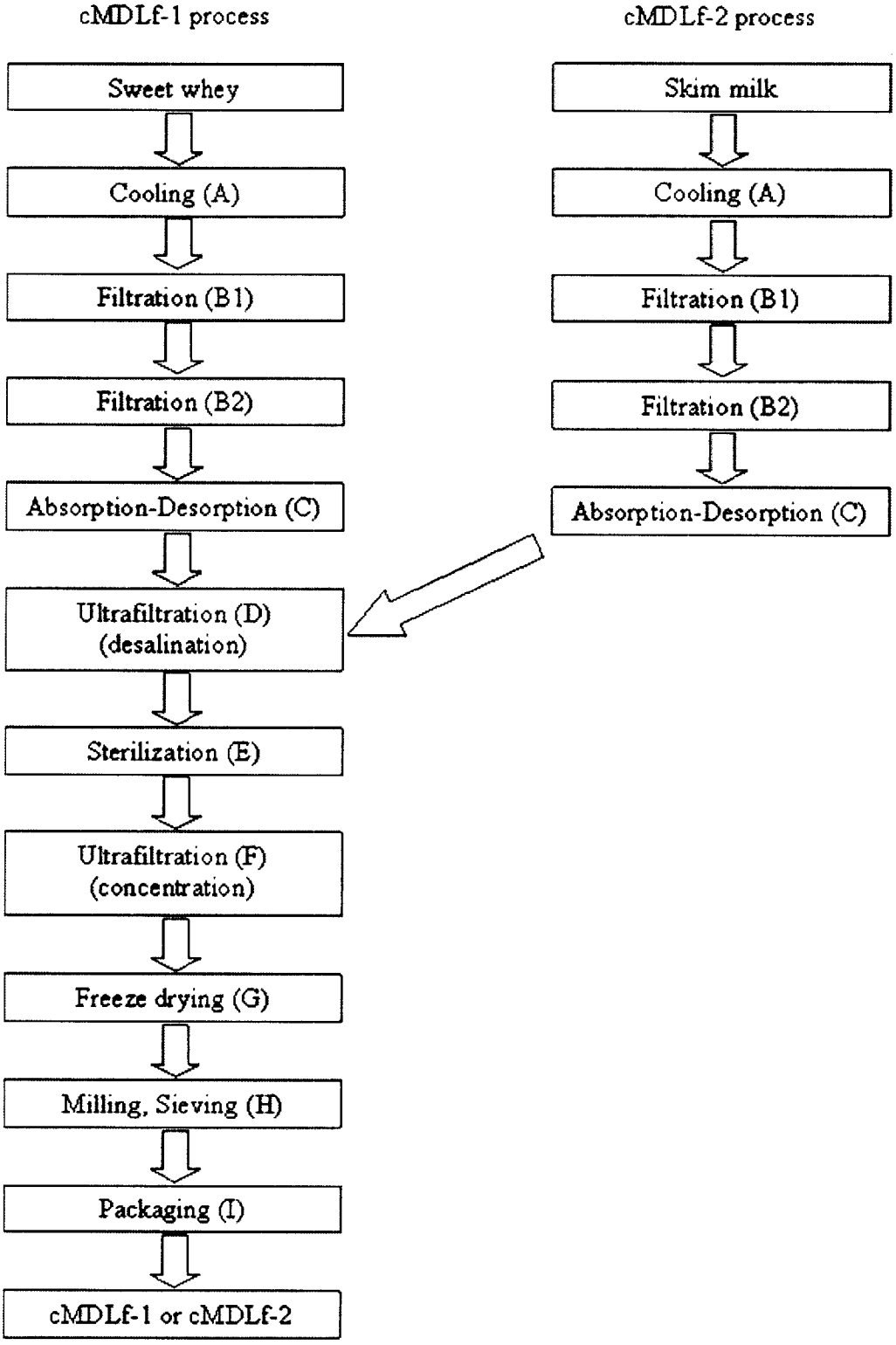


**Figure 2: Production Process of Cow's Milk-Derived Lactoferrin**

## 2. STARTING MATERIALS AND PROCESSING AIDS

Either sweet whey or skim milk is used as the starting material for the production of cMDLf. Both skim milk and sweet whey conform to the European Union Food Hygienic Guidelines and The German Food Law ("Lebensmittel-und Bedarfsgegenstaendegesetz" - LMBG). Besides demineralized water, all materials used in the production process are food grade (Table 2).

**Table 2. Processing Aids and Chemicals Used in the Production of Cow's Milk-Derived Lactoferrin (cMDLf)**

| Process Aid or Chemical | Manufacturer | |
|---|---|---|
| | At Milei for cMDLf-1, cMDLf-2 | At Riedlingen for cMDLf-2 |
| Demineralized water | Milei | Riedlingen plant |
| Sodium chloride (NaCl) | Herkommer & Bangerter | Herkommer & Bangerter |
| Hydrochloric Acid (HCl) | Herkommer & Bangerter | Not used |
| CM Sephadex C-50 or SP Sepharose Big Beads | GE Healthcare | GE Healthcare |
| Filter cloth (1um) | Wolftechnik Filtersysteme | Wolftechnik Filtersysteme |
| Filter cloth (5um) | Wolftechnik Filtersysteme | Not used |
| GR61PP Membrane | Alfa Laval | Not used |

# C. FINISHED PRODUCT SPECIFICATIONS

## 1. PRODUCT SPECIFICATIONS AND BATCH RECORDS

The manufacturing specifications for cMDLf are listed in Table 3. The product consists of 94.5% protein, 4.2% moisture and 1.3 % ash, and lactoferrin accounts for 96% of the protein fraction. The pH of the finished product ranges between 5.5 and 7.2 with a water activity ($a_w$) of less than 0.2. Complete solubility is achieved when 2 g of lactoferrin are added to 100 ml of 20°C water. The bound iron content is less than 35 mg/100 g. Because cMDLf has two iron-binding sites, the iron binding capacity of the final product is ≥75%[1]. Batch analyses of three non-consecutive lots of cMDLf from each source [sweet whey (cMDLf-1) and skim milk (cMDLf-2)] are provided in Table 4 and confirm that the production processes is capable of generating highly purified cMDLF.

The amount of lactoferrin in cMDLF-1 and cMDLF-2 is determined by the Kjeldahl method (N x 6.38) and HPLC, and verified by absorption at 280 nM. Analytical methods for the other specifications include the AOAC official method 905.02 for atomic absorption spectrometry for iron content, ICP-MS for heavy metals, microbial analyses for total aerobic count, coliform bacteria, yeast and mold, coagulase positive *staphylococci* and *salmonella*, for *Bacillus cereus*, and for *Enterobacter sakazakii*, GC-MS for PCBs, GC for pesticides, HPLC-MS and microbial quantification for antibiotics, HPLC for aflatoxin M1 and Germanium semiconductor detector for radioactivity. Parasiticides are also screened by HPLC.

[1] Theoretically, each lactoferrin can bind two irons with a maximum iron binding of 2x56 g iron/80,000 g lactoferrin = 140 mg/100g. Therefore, the calculated binding capacity of cMDLf product is ≥1-35/140 = 75%.

| Table 3. Manufacturing Specifications for Cow's Milk-Derived Lactoferrin (cMDLf) | | |
|---|---|---|
| **Specifications** | **Limits** | **Method** |
| Appearance | Pink and odorless powder | Visual check |
| Foreign matter | Absent | Visual checking in 10 g |
| pH (2% solution) | 5.2 – 7.2 | pH meter |
| Protein content (%) | ≥ 94.5 | Kjeldahl method (n x 6.38) |
| cMDLf Purity (%/protein) | ≥ 96.0 | HPLC |
| Fat (% dry weight) | ≤ 0.5 | AOAC 905.02 |
| Residue on ignition (%) | ≤ 1.3 | 500-550°C for 1-2 d |
| Loss on drying (%) | ≤ 4.2 | Heat to 105°C, 5 hours |
| Water Activity ($a_w$) [g] | ≤ 0.2 | Water activity test device |
| Iron content (mg/100 g) | ≤ 35 | Atomic absorption spectrometry |
| Iron saturation (%) | ≤ 25 [a] | Calculated iron content |
| Heavy Metals | | |
| *Lead (pb) ppm* | ≤ 1 | ICP-MS[b] |
| *Cadmium (Cd) ppm* | ≤ 0.05 | ICP-MS[b] |
| *Mercury (Hg) ppm* | ≤ 0.05 | ICP-MS[b] |
| *Arsenic (as $As_2O_3$) ppm* | ≤ 1 | Fluorescent X ray analysis |
| Solubility | | |
| In water (2% at 20°C) (%) | = 100 | Visual check |
| Transmittance (2% solution, 600nm) (%) | ≥80 | Spectrophotometer |
| Microbiological tests | | |
| *Total aerobic count* | ≤ 1,000 cfu/g | Standard Agar |
| *Coliform bacteria* | Negative/0.1 g | Desoxycholate Agar |
| *Coagulase positive staphylococci* | Negative/g | Mannitol salt agar with egg yolk |
| *Yeast and Mold* | ≤ 10 cfu /g | Potato dextrose agar |
| *Salmonella* | Negative/25 g | DHL agar |
| *Bacillus cereus* [g] | Negative in 20 mg | NGKG agar plate |
| *Enterobacter sakazakii* [g] | Negative in 333 g | Violet red bile dexystrose agar |
| PCBs (mg/kg)[g] | <0.1 | GC-MS[c] |
| Pesticides (mg/kg)[g] | <0.1 | Gas chromatography[d] |

| Table 3. Manufacturing Specifications for Cow's Milk-Derived Lactoferrin (cMDLf) | | |
|---|---|---|
| **Specifications** | **Limits** | **Method** |
| Antibiotics[g] | | |
| *Dihydrostreptomycin, streptomycin* | Not detected [e] | Microbial quantification method |
| *Cefazolin, ampicillin, chloramphenicol* | Not detected [e] | HPLC-MS |
| Aflatoxin M1 (μg/kg)[g] | <0.5 | HPLC method |
| Radioactivity ($^{134}$Cs + $^{137}$Cs) (Bq/kg)[g] | < 100 | Germanium semiconductor detector[f] |

Notes:

a. Theoretically, each lactoferrin can bind two irons with a maximum iron binding of 2x56 g iron/80,000 g lactoferrin = 140 mg/100 g. Therefore, the calculated binding capacity of cMDLf product is ≥1-35/140 = 75%.
b. According to the Method of Analysis in Health Science (2010).
c. PCBs include PCB #28, #52, #101, #118, #153, #138, #180. The detection limit is 0.01 ng/g.
d. Pesticides include BHC, DDT, aldrin, dieldrin, endrin, heptachlor and hexachlorobenzene. The detection limit for BHC, DDT, heptachlor and hexachlorobenzene is 0.01 ppm. The detection limit for aldrin, dieldrin and endrin is 0.005 ppm.
e. The detection limit for dihydrostreptomycin and streptomycin is 0.02 ppm. The detection limits for cefazolin, ampicillin and chloramphenicol are 0.01 ppm, 0.005 ppm and 0.0005 ppm, respectively.
f. The detection limits for $^{134}$Cs and $^{137}$Cs are 5.4 Bq/kg and 6.3 Bq/kg (1,000 sec) respectively.
g. Analyzed quarterly.

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| Table 4. Batch Analyses of Cow's Milk-Derived Lactoferrin (cMDLf) | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Item** | **Specification Limits** | **Lot # (cMDLf-1 from Sweet Whey)** | | | **Lot # (cMDLf-2 from Skim Milk)** | | |
| | | **131110** | **151110** | **171110** | **101110** | **161210** | **211210** |
| Appearance | Pink and odorless powder | Conform | Conform | Conform | Conform | Conform | Conform |
| Foreign matter | Absent | Conform | Conform | Conform | Conform | Conform | Conform |
| pH (2% solution) | 5.2 - 7.2 | 5.53 | 5.58 | 5.75 | 5.50 | 5.59 | 5.20 |
| Protein content (% dry weight) | ≥ 94.5 | 98.8 | 99.4 | 99.3 | 99.2 | 98.8 | 98.7 |
| cMDLf Purity (per protein) | ≥96.0 | 97.3 | 96.8 | 96.8 | 97.0 | 97.7 | 97.2 |
| Fat (% dry weight) | ≤ 0.5 | <0.5[a] | <0.5[a] | <0.5[a] | <0.5[a] | <0.5[a] | <0.5[a] |
| Residue on ignition (% dry weight) | ≤1.3 % | 0.13 | 0.17 | 0.11 | 0.07 | 0.09 | 0.05 |
| Loss on drying (%) | ≤4.2% | 0.41 | 0.05 | 0.35 | 0.54 | 0.70 | 0.43 |
| Water Activity ($a_w$) | ≤0.2 | 0.03 | 0.03 | 0.03 | 0.02 | 0.03 | 0.03 |
| Iron content (mg/100 g) | ≤ 35 mg/100 g | 21.1 | 21.7 | 19.7 | 8.20 | 9.59 | 9.51 |
| *Iron saturation (%)* | ≤ 25 | 15.1 | 15.5 | 14.1 | 5.9 | 6.9 | 6.8 |
| Minerals | | | | | | | |
| *Sodium (na) (mg/100 g)* | | 38.0 | 39.9 | 42.0 | 33.5 | 47.1 | 48.2 |
| *Potassium (K) (mg/100 g)* | | 1.00 | 5.36 | 5.30 | 1.09 | 1.11 | 2.70 |
| *Magnesium (Mg) (mg/100 g)* | | 0.49 | 0.51 | 0.55 | 0.56 | 0.55 | 0.53 |
| *Phosphorus (p) (mg/100 g)* | | 3.25 | 3.87 | 3.99 | 2.95 | 3.14 | 3.57 |
| *Calcium (Ca) (mg/100 g)* | | 8.14 | 8.87 | 9.15 | 7.81 | 7.93 | 7.69 |
| *Chlorine (Cl) (mg/100 g)* | | 766 | 816 | 758 | 795 | 764 | 889 |
| *Copper (Cu) (mg/100 g)* | | 0.28 | 0.09 | 0.31 | ND[b] | ND[b] | ND[b] |
| *Zinc (Zn) (mg/100 g)* | | 0.15 | 0.64 | 0.32 | 0.32 | 0.29 | 0.25 |
| *Manganese (Mn) (mg/100 g)* | | ND[b] | ND[b] | ND[b] | 0.01 | 0.01 | 0.01 |
| Heavy Metals | | | | | | | |
| *Lead (pb) ppm* | ≤ 1 | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] |
| *Cadmium (Cd) ppm* | ≤ 0.05 | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] |
| *Mercury (Hg) ppm* | ≤ 0.05 | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] |
| *Arsenic (as $As_2O_3$) ppm* | ≤ 1 | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] |
| Solubility | | | | | | | |
| In water (2% at 20°C) (%) | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Transmittance (2% solution, 600 nm) (%) | ≥80% | 91.9 | 92.0 | 90.6 | 81.4 | 95.2 | 85.7 |

0000021

**Table 4. Batch Analyses of Cow's Milk-Derived Lactoferrin (cMDLf)**

| Item | Specification Limits | Lot # (cMDLf-1 from Sweet Whey) | | | Lot # (cMDLf-2 from Skim Milk) | | |
|---|---|---|---|---|---|---|---|
| | | 131110 | 151110 | 171110 | 101110 | 161210 | 211210 |
| Microbiological tests | | | | | | | |
| *Total aerobic count* | ≤ 1,000 cfu/g | 0 | 0 | 0 | 0 | 0 | 0 |
| *Coliform bacteria* | Negative/0.1 g | Negative | Negative | Negative | Negative | Negative | Negative |
| *Coagulase positive staphylococci* | Negative/g | Negative | Negative | Negative | Negative | Negative | Negative |
| *Yeast and Mold* | ≤ 30 cfu/g | 0 | 0 | 0 | 0 | 0 | 0 |
| *Salmonella* | Negative/25 g | Negative | Negative | Negative | Negative | Negative | Negative |
| *Bacillus cereus* | Negative in 20 mg | Negative | Negative | Negative | Negative | Negative | Negative |
| *Enterobacter sakazakii* | Negative in 333 g | Negative | Negative | Negative | Negative | Negative | Negative |
| PCBs | < 0.1 mg/kg | ND[d] | NT | NT | ND[d] | NT | NT |
| Pesticides | < 0.1 mg/kg | ND[e] | NT | NT | ND[e] | NT | NT |
| Antibiotics | | | | | | | |
| *Dihydrostreptomycin, streptomycin* | Not detected | ND[f] | NT | NT | ND[f] | NT | NT |
| *Cefazolin, ampicillin, chloramphenicol* | Not detected | ND[g] | NT | NT | ND[g] | NT | NT |
| Aflatoxin M1 | < 0.5 μg/kg | ND[h] | NT | NT | ND[h] | NT | NT |
| Radioactivity ($^{134}Cs + ^{137}Cs$) | < 100 Bq/kg | NT | ND[i] | NT | NT | ND[i] | NT |

Notes:

a. Detection limit for fat content is 0.5%.
b. Detection limits are 0.05 mg/100 g for copper and 0.003 mg/100 g for manganese.
c. Detection limits are 0.1 μg/g for Pb, 0.01 μg/g for Cd, 0.01 μg/g for Hg and 1 μg/g for $As_2O_3$.
d. PCBs tested include PCB #28, #52, #101, #118, #153, #138, #180. The detection limit for each PCB is 0.01 ng/g.
e. Pesticides tested include BHC, DDT, aldrin, dieldrin, endrin, heptachlor and hexachlorobenzene. The detection limit is 0.01 ppm for BHC, DDT, heptachlor and hexachlorobenzene and is 0.005 ppm for aldrin, dieldrin and endrin.
f. Detection limit is 0.02 ppm.
g. Detection limit is 0.01 ppm for cefazolin, 0.005 ppm for ampicillin, 0.0005 ppm for chloramphenicol.
h. Detection limit is 0.5 ppb.
i. Detection limits are 6.3 Bq/kg for $^{137}Cs$ and 5.4Bq/kg for $^{134}Cs$.

ND stands for "not detected", NT stands for "not tested" as these parameters are monitored periodically only.

0000022

## 2. PATHOGENIC BACTERIA

cMDLf was analyzed for the presence of *Listeria monocytogenes, Clostridium perfringens, Cronobacter (Enterobacter) sakazakii* and *Yersinia enterocolytica* because they are potential contaminants of raw milk. None of these pathogenic bacteria were detected (Table 5).

**Table 5. Batch Analysis of Pathogenic Bacteria in Cow's Milk-Derived Lactoferrin (cMDLf)**

| Microorganism | Lot # (cMDLf-1) | | | Lot # (cMDLf-2) | | |
|---|---|---|---|---|---|---|
| | 131110 | 151110 | 171110 | 101110 | 161210 | 211210 |
| *Listeria monocytogenes*[a] | Neg | Neg | Neg | Neg | Neg | Neg |
| *Clostridium perfringens*[b] | Neg | Neg | Neg | Neg | Neg | Neg |
| *Yersinia enterocolytica*[c] | Neg | Neg | Neg | Neg | Neg | Neg |

cMDLf-1is derived from sweet whey and cMDLf-2 is derived from skim milk.
Neg stands for "negative."

a. *Listeria monocytogenes* was tested with Palukam Agar (by the Ministerial Ordinance Concerning Compositional Standards, etc. for milk and milk products in Japan). Limit is negative in 25 g.
b. *Clostridium perfringens* was tested with the method described in the Microbes part, Food Hygiene Inspection Guideline. Limit is negative in 30 g.
c. *Yersinia enterocolytica* was tested with the method described in the Microbes part, Food Hygiene Inspection Guideline. Limit is negative in 25 g.

## 3. PARASITICIDES

cMDLf was analyzed for the presence of parasiticides including abamectin, doramectin, moxidectin, eprinomectin (as eprinomectin $B_{1a}$) and ivermectin (as 22, 23-dihydroavermectin $B_{1a}$) because they are potential contaminants of raw milk. None of these parasites were detected (Table 6).

**Table 6. Batch Analysis of Parasiticides in Cow's Milk-Derived Lactoferrin (cMDLf)**

| Parasiticide | Lot # 131110 (cMDLf-1) | Lot # 101110 (cMDLf-2) |
|---|---|---|
| Abamectin | Not detected | Not detected |
| Doramectin | Not detected | Not detected |
| Moxidectin | Not detected | Not detected |
| Eprinomectin (as Eprinomectin $B_{1a}$) | Not detected | Not detected |
| Ivermectin (as 22, 23-dihydroavermectin $B_{1a}$) | Not detected | Not detected |

cMDLf-1 is derived from sweet whey and cMDLf-2 is derived from skim milk.
Parasiticides are analyzed by HPLC. The detection limits are 0.005 ppm

000023

### 4. OTHER WHEY PROTEINS

The levels of bovine serum albumin, IgG, β-lactoglobulin, lactoperoxidase, and angiotensin in cMDLf-1 and cMDLf-2 were determined by HPLC. The amount of bovine serum albumin, IgG, β-lactoglobulin, and lactoperoxidase were negligible in both products. Angiogenin was found at low levels (1-2% peak area) in both products, but the ratio of angiogenin to lactoferrin in cMDLf-1 and cMDLf-2 is similar to that found in cow's milk. Thus, it is reasonable to assume that other milk proteins are not selectively concentrated during the production process. Casein, the main protein in milk, was not seen in either lactoferrin product.

## D. STABILITY OF CMDLF

cMDLf products were packaged in polyethylene or aluminum bags, the standard materials for the storage of cMDLf. Appearance, flavor, solubility, moisture content, pH of a 2% solution, lactoferrin stability, microbial contamination and growth were monitored periodically. cMDLf stability was quantified by HPLC (purified product) and by an agglutination immunoassay and/or enzyme-linked immunosorbent assay (in food matrices) (Yamauchi et al., 2004). When incubated at room temperature, cMDLf was stable for up to 96 mo when packaged in polyethylene bags and stable for up to 102 mo when packaged in aluminum bags. cMDLf was stable for up to 3 yr in powdered infant formulas and up to 46 mo in skim milk powder. In yogurt products maintained at less than 10°C, cMDLf was stable for up to 17 d.

# III. INTENDED EFFECT

The intended effect is to increase the intake of cMDLf from consumption of cow's milk-based infant formulas and other cow's milk-based products and chewing gum.

In the United States, cMDLf has been determined safe and GRAS for use as an ingredient in sport and functional foods at concentrations of 100 mg/serving (GRN 77). cMDLf has also been determined safe and GRAS for use as a component of an antimicrobial spray for application to uncooked beef (GRN 67 and GRN 130). Because cMDLf is naturally present in small quantities in cow's milk, products derived from this source will not contain very much of this protein.

Lactoferrin is a member of the transferrin family of iron-binding glycoproteins and is found in mucosal secretions, such as milk and saliva, and the secondary granules of neutrophils (Baggiolini, 1972; Masson and Heremans, 1971; Masson et al., 1966). Lactoferrin from cows (*Bos taurus*) is 69% homologous to human lactoferrin, and although cMDLf contains many of the same structural features as human lactoferrin, it contains approximately four times more iron than human lactoferrin (Crichton, 1990; Pierce et al., 1991; Baker et al., 1994; Wang et al., 1984). The lactoferrin level in human milk is approximately 2 mg/ml milk (GRN 235; Lien et al., 2004; Prentice, 1995). Cow's milk contains approximately 0.1 mg lactoferrin/ml milk (GRN 77).

As a natural defense protein, lactoferrin is known for its ability to inhibit the growth of certain strains of pathogenic bacteria by scavenging iron (reviewed in Adlerova et al., 2008). Some cell culture experiments indicate an inhibitory effect of cMDLf on osteoclasts (Cornish et al., 2004; Blais et al., 2009; Yamano et al., 2010), plus multiple stimulatory effects of cMDLf on osteoblasts (Cornish et al., 2004; Cornish et al., 2006; Takayama and Mizumachi, 2008; Blais et al., 2009). Other studies suggest that oral ingestion of cMDLf may be beneficial for maintaining iron homeostasis in infants and women (Chierici et al., 1992; Koikawa et al., 2008; Nappi et al., 2009; Paesano et al., 2006; Paesano et al., 2009; Paesano et al., 2010).

# IV. INTENDED USE, HISTORY OF USE, AND ESTIMATED DAILY INTAKE

## A. INTENDED USE

Milk, by definition is the lacteal secretion practically free of colostrum obtained by the milking of one or more healthy cows (21CFR131.110). Morinaga Milk Industry Co., Ltd. intends to use cMDLf as an ingredient in cow's milk-based infant formulas as well as selected uses in general foods including powdered milks, yogurts, ice creams and sherbets, and chewing gums. The product categories to which cMDLf will be added and the corresponding maximum use-levels are summarized in Table 7.

**Table 7. Intended Uses of Cow's Milk-Derived Lactoferrin (cMDLf) and Its Maximum Use Levels**

| Food group[a] | Foods with cMDLf added | Maximum use level |
|---|---|---|
| Yogurt | All yogurt products including yogurt drinks and those as baby foods. | 100 mg cMDLf/100 g |
| | Non-fermented milk fortified with probiotics in addition to lactoferrin.[e] | |
| Powdered milk | All powdered, not reconstituted, mixtures with dry milk | 400 mg cMDLf/100 g |
| Infant formula | Ready-to-feed formulas | 13 mg cMDLf/100 ml[b] |
| | Liquid concentrates, not reconstituted | 26 mg cMDLf/100 ml[c] |
| | Powdered infant formulas (dry, not reconstituted) | 100 mg cMDLf/100 g[d] |
| Milk dessert | Ice creams and sherbets | 200 mg cMDLf/100 g |
| Sugars and sweets | Chewing gums | 30 mg cMDLf/g |

a. Foods are grouped based on the US Food and Nutrient Database for Dietary Studies (FNDDS).
b. The maximum cMDLf concentration in ready-to-feed infant formulas is 13 mg/100 ml, equivalent to 12.26 mg/100 g because a 100 ml ready-to-feed infant formula weighs approximately 106 g.
c. Normally, liquid concentrate is prepared by diluting with water at 1:1 ratio before feeding.
d. The maximum cMDLf concentration in infant formula powders is 100 mg/100 g. When powdered infant formulas are reconstituted by mixing 13 g powder with 93 g water yielding 106 g formula (approximately 100 ml), the reconstituted infant formulas, therefore, contain supplemental cMDLf at a concentration of approximately 13 mg/100 ml (or 12.26 mg/100 g).
e. Non-fermented milk fortified with probiotics in addition to cMDLf is new to US consumers. Yogurt drinks were used as surrogate products for the intake estimate for cMDLf.

Spherix Consulting has completed an assessment of the consumption of cMDLf by the U.S. population resulting from background exposure and proposed uses of cMDLf. Estimates for the intake of cMDLf were based on the cMDLf containing foods and average background levels, proposed food uses and maximum supplemental levels in conjunction with food consumption data included in the National Center for Health Statistics' (NCHS) 2007-2008 National Health and Nutrition Examination Surveys (NHANES) (CDC, 2006; USDA, 2010; Bodner-Montville et al., 2006). The mean and 90$^{th}$ percentile all-user intakes were calculated.

## B. HISTORY OF USE

### 1. EXPOSURE TO HUMAN LACTOFERRIN

Lactoferrin is a naturally occurring protein present in mucosal secretions, ie. milk and saliva, and the secondary granules of neutrophils. During infection and inflammation, lactoferrin is released from neutrophils and thus, is found in the plasma.

Human lactoferrin is encoded by one gene, located on chromosome 3 (Taylor et al., 2004). It is generally accepted that human milk-derived and neutrophil-derived lactoferrin differ only in the composition of their carbohydrate side chains; human milk-derived lactoferrin (hMDLf) contains fucose residues whereas neutrophil-derived lactoferrin does not (Teng, 2002; Derisbourg et al., 1990; Taylor et al., 2004). Functionally, both types of lactoferrin appear to be equivalent (Wu et al., 1995; Broxmeyer et al., 1986) and no reports have investigated how the carbohydrate side chains and/or their composition contributes to lactoferrin immunogenicity. Infants and adults can be exposed to endogenous/neutrophil-derived lactoferrin, but the first exposure to exogenous lactoferrin is through consumption of breast milk and/or milk-based infant formula. Although the amount of lactoferrin in human milk varies from country to country (Figure 3), the mean levels of lactoferrin are approximately 5 mg/ml in colostrum, 2.5 mg/ml in mature milk (day 15 to 84 postpartum), and 1 mg/ml in mature milk up to two yr postpartum (GRN 235; Lien et al., 2009, Prentice, 1995). Cow's milk-based infant formulas contain approximately 0.1 to 0.2 mg cMDLf/ml formula or 101 to 210 ppm.

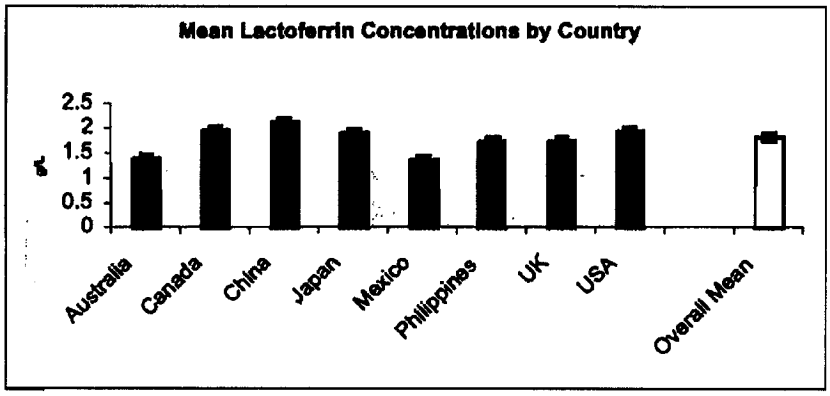


**Figure 3: Mean lactoferrin concentrations by country (Lien et al., 2009)**
**Mature milk was collected from women who had delivered full-term healthy infants and analyzed by HPLC.**

## 2. EXPOSURE TO cMDLf

Humans have consumed cow's milk for centuries. Milk is composed of a complex mixture of lipids, proteins, carbohydrates, vitamins, and minerals. Nutrition values of milk on the market are available at USDA's web site, USDA National Nutrient Database for Standard Reference (http://www.nal.usda.gov/fnic/foodcomp/search/). The average composition of whole milk consists of 86.6% water, 4.1% fat, 3.6% protein, 5.0% lactose, and 0.7% ash (Swaisgood, 1985).

Milk protein is a combination of caseins and whey proteins. Their average concentrations and relative amounts are shown in Table 8. Caseins account for 79.2% (27 mg/ml milk) of the total milk proteins. The remaining 20.5% (7.0 mg/ml milk) is whey protein. cMDLf, which is a whey protein, accounts for 0.3% (0.1 mg/ml milk) of the total protein or 1.4% of the whey protein (Barth and Behnke, 1997).

**Table 8. Concentration of Proteins in Cow's Milk**

| Protein components | | Absolute concentration | Relative concentration to total milk protein |
|---|---|---|---|
| Whey proteins | Lactoferrin | 0.1 mg/ml milk | 0.3% |
| | Others | 7.0 mg/ml milk | 20.5% |
| Caseins | | 27.0 mg/ml milk | 79.2% |
| Total milk protein | | 34.1 mg/ml milk | 100% |
| Note: Adapted from (Barth and Behnke, 1997) | | | |

cMDLf-supplemented infant formulas were first introduced to the market in Japan in 1986 in a product named "BF-L" by the Morinaga Milk Industry Co., Ltd. BF-L was intended to be used 0- to 9-mo-old and contained 50 mg of cMDLf/100 g powered milk. The cMDLf level was then increased to 80 mg/100 g powdered milk to be consistent with the levels used in competitors' products and the new product was named "Hagukumi". Three yr later, Morinaga launched "Chil-Mil" a follow-up infant formula for toddlers of 9 mo to 3 yr, which contains cMDLf at 45 mg/100 g powered milk. All regulatory approval documents for Morinaga Milk products containing cMDLf are provided in Appendix 1. Morinaga has subsequently introduced and sold cMDLf-supplemented infant formulas and follow-up formulas in Taiwan (2000), Pakistan (2001), mainland China (2004), and Indonesia (2004). Infant formulas containing cMDLf are also sold by other companies in Japan, Korea, and China.

Since the release of cMDLf-containing formulas, Morinaga has sold approximately 3,200 metric tons and 3,500 metric tons annually for the 0- to 9-month formulation and 9-month to 3-yr formulation in Japan. With consumption of Morinaga's cMDLf-supplemented formulas by over a million infants and toddlers in Japan since 1986, there have been no reported significant health problems, including allergenic reactions attributable to cMDLf, associated with either of these two products containing cMDLf based on post marketing surveillance by Morinaga.

Although current sales volumes are lower in Pakistan (700 and 500 metric tons/yr for 0- to 6-mo and 6-mo to 3-yr formulas, respectively), Indonesia (sales volume not available), and China (sales volume not available), no heath problems, including allergenic reactions, have been reported based on post marketing surveillance by Morinaga. Information on sales volume and health status for Korea are not available.

Both cMDLf-supplemented infant formulas sold in Japan by Morinaga Milk Industry Co., Ltd. have been certified by the Japanese Ministry of Health as Special Nutritious Foods according to the Nutrition Improvement Law (Appendix 2). The specification monographs for these two products are found in Appendix 1-A. Morinaga's cMDLf has been listed on the "natural additive list" in Japan since 1989 and was added to the "existing additive list" in Japan in 1995 (http://www.ffcr.or.jp/zaidan/ffcrhome.nsf/pages/list-exst.add) (Appendix 1-B). In Japan, there is no specific restriction for cMDLf because it is considered a natural material. Regulatory approval documents for the use of cMDLf in infant formulas and follow-up formulas in China (since 2004) and for the use of cMDLf as a food additive in Taiwan (since 2000) and in Korea are provided in Appendices 1-C, 1-D and 1-E, respectively.

**a) Background exposure to cMDLf from the consumption of cow's milk based infant formulas**

Breast milk is recognized as the standard for infant nutrition. Infant formulas are patterned after breast milk to provide the right quality and balance of protein, fat, and carbohydrate for the first 12 mo of life to promote normal growth and development.

Infant formula intakes were estimated using data from the U.S. NHANES 2007-2008 survey. Formula-fed infants (all-user) ages 0- to 4- and 5- to 11-mo-old consumed an average of 818 g (liquid; 143g/kg bw/day) and 713 g (liquid; 82 g/kg bw/day) infant formula/day, respectively. Infant formula users (all-user) ages 12- to 35-mo-old consumed an average of 593 g (liquid; 61 g/kg bw/day) infant formula/day.

There are a number of infant formula brands on the US market including Enfamil, Similac, Good Start, as well as a number of store brands. Nutritional compositions of infant formulas are available online at Self Nutrition Data (http://nutritiondata.self.com/). Although infant formulas are available in the forms of powder, liquid concentrate and ready-to-feed, their nutritional compositions are similar. They are formulated with or without iron, and supplemented with or without arachidonic acid and docosahexanoic acid. In addition, infant formulas can be either cow's milk-based or soy protein-based. For cow's milk-based infant formulas, protein levels range between 1 to 2 g/100 g ready-to-feed infant formula or 6 to 12 Kcal protein/100 Kcal formula (based on 4 Kcal/g protein and approximately 66 Kcal/100 g ready-to-feed infant formula).

The protein composition of infant formula varies from manufacturer to manufacturer. For cow's milk-based infant formulas, milk protein in Similac (Abbott Labs) is composed of approximately 48% whey protein and 52% casein, while the ratio of whey protein to casein in Enfamil (Mead Johnson) and Good Start (nestle Carnation) is 60:40 and 100:0, respectively (Nutrition Services, 2009). Because cMDLf accounts for approximately 1.4% of whey protein (Barth and Behnke, 1997), the amount of cMDLf available from these infant formula ranges between 101 to 210 ppm (Table 9).

Using the infant formula consumption data and estimates of cMDLf concentration in ready-to-feed infant formulas, we found that formula-fed-infants (all-user) 0- to 4-mo-old consumed between 82.6 to 171.8 mg lactoferrin/day (14.4 to 30 mg/kg bw/day) (Table 9). cMDLf intake by infants 5- to 11-mo-old ranged from 72 to 149.7 mg/day (8.3 to 17.2 mg/kg bw/day). The exposure of infant formula users 12- to 35-mo-old ranged from 59.9 to 124.5 mg/day (6.2 to 12.8 mg/kg bw/day).

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**Table 9. Infant Formula-User Background Estimated Daily Intake (EDI) of Cow's Milk-Derived Lactoferrin (cMDLf) from Consumption of Cow's Milk-Based Infant Formulas**
**(Infant and Toddler Groups; 2007-2008 NHANES Data)**

| Product | | Similac | Enfamil | Good Start |
|---|---|---|---|---|
| Manufacturer | | Abbott Labs | Mead Johnson | Nestle Carnation |
| Protein concentration [a, b] | | 1.5 g/100 g | 1.5 g/100 g | 1.5 g/100 g |
| Ratio of whey protein to casein | | 48:52 | 60:40 | 100:0 |
| cMDLf concentration in the formula [c] | | 101 ppm | 126 ppm | 210 ppm |
| Infants of 0-4 mo [d] | Percent users | 98.1% | | |
| | Average intake | 82.6 mg/d (14.4 mg/kg/d) | 103.1 mg/d (18 mg/kg/d) | 171.8 mg/d (30 mg/kg/d) |
| | 90th percentile | 119.9 mg/d (21.7 mg/kg/d) | 149.6 mg/d (27.1 mg/kg/d) | 249.3 mg/d (45.2 mg/kg/d) |
| Infants of 5-11 mo [e] | Percent users | 78.7% | | |
| | Average intake | 72 mg/d (8.3 mg/kg/d) | 89.8 mg/d (10.3 mg/kg/d) | 149.7 mg/d (17.2 mg/kg/d) |
| | 90th percentile | 106.3 mg/d (12.1 mg/kg/d) | 132.6 mg/d (15.1 mg/kg/d) | 220.9 mg/d (25.2 mg/kg/d) |
| Toddlers of 12-35 mo [f] | Percent users | 2.6% | | |
| | Average intake | 59.9 mg/d (6.2 mg/kg/d) | 74.7 mg/d (7.7 mg/kg/d) | 124.5 mg/d (12.8 mg/kg/d) |
| | 90th percentile | 92.4 mg/d (10.2 mg/kg/d) | 115.3 mg/d (12.7 mg/kg/d) | 192.2 mg/d (21.2 mg/kg/d) |

a. Nutritional composition is derived from ready-to-feed infant formulas.
b. Protein level in an infant formula ranges between 1 g and 2 g/100 g, with an average of 1.5% (http://nutritiondata.self.com/). 100 ml ready-to-feed infant formula weighs approximately 106 g.
c. Lactoferrin accounts for approximately 1.4% of whey protein (Barth and Behnke, 1997).
d. Formula-fed infants (all-user) of 1- to 4-mo consumed an average of 818 g (liquid; 143 g/kg bw/d) infant formula/day and 1187 g/d (215 g/kg bw/d) for heavy users (90th percentile) as calculated using data from the U.S. NHANES 2007-2008 survey.
e. Formula-fed infants (all-user) of 5- to 11-mo-old consumed an average of 713 g (liquid; 82 g/kg bw/d) infant formula/day and 1052 g/d (120 g/kg bw/d) for heavy users (90th percentile) as calculated using data from the U.S. NHANES 2007-2008 survey.
f. Infant formula users (all-user) aged between 12- to 35-mo-old consumed an average of 593 g (liquid; 61g/kg bw/d) infant formula/day on average and 915 g/d (101 g/kg bw/d) for heavy users (90th percentile) as calculated using data from the U.S. NHANES 2007-2008 survey.

**b) Background exposure to cMDLf from the consumption of milk and milk products excluding infant formulas**

The average lactoferrin content in cow's milk is approximately 0.1 mg/ml (Barth and Behnke, 1997); which means an 8-oz glass of milk would contain about 23 mg of lactoferrin. Using the food consumption data of NHANES 2007-2008, Spherix determined the average intake of cMDLf from the consumption of cow's milk and milk products and presented both absolute (mg/day) and weight-based intakes (mg/kg bw/day) in Table 10. Cow's milk and milk products used for estimating the background exposure to cMDLf include all fluid and flavored milks (excluding imitation milk), powdered, concentrated and reconstituted milks, yogurts, milk-based meal replacements, milk deserts/sauces/deserts, and cheeses (excluding imitation cheeses). On average, the background exposure to cMDLf from the consumption of milk and milk products was 39 mg/day (0.8 mg/kg bw/day). For heavy consumers (90$^{th}$ percentile), the background exposure to cMDLf was 79 mg/day (1.9 mg/kg bw/day). On an individual population basis, the greatest background exposure to cMDLf from the consumption of milk and milk products was found to occur in toddlers at 59 mg/day (4.8 mg/kg bw/day) on average and at 105 mg/day (8.6 mg/kg bw/day) for heavy consumers (90$^{th}$ percentile).

**Table 10. All-User Estimated Daily Intake (EDI) of Cow's Milk-Derived Lactoferrin (cMDLf) from Background Exposure to Milk and Milk Products Excluding Infant Formulas**
**(U.S Population Groups; 2007-2008 NHANES Data)**

| Population | $N_u/N_p$ [a, b] | Percent users[c] | Absolute EDI (mg cMDLf/d) | | Weight-based EDI (mg cMDLf/kg bw/d) | |
|---|---|---|---|---|---|---|
| | | | Mean | 90$^{th}$ Percentile | Mean | 90$^{th}$ Percentile |
| Infants, 0-4 mo | 2/132 | 0.8 | 0.5 | 1.5 | 0.1 | 0.2 |
| Infants, 5-11 mo | 97/221 | 44.8 | 31 | 78 | 3.4 | 9.0 |
| Toddlers, 12-35 mo | 437/446 | 97.6 | 59 | 105 | 4.8 | 8.6 |
| Children, 3-11 yr | 1310/1335 | 97.7 | 45 | 79 | 1.8 | 3.5 |
| Teen, 12-19 yr | 863/963 | 90.4 | 44 | 96 | 0.7 | 1.5 |
| Adult, 20+ yr | 4045/4628 | 88.7 | 36 | 74 | 0.5 | 1.0 |
| Total population | 6754/7725 | 89.4 | 39 | 79 | 0.8 | 1.9 |

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**Table 10. All-User Estimated Daily Intake (EDI) of Cow's Milk-Derived Lactoferrin (cMDLf) from Background Exposure to Milk and Milk Products Excluding Infant Formulas**
**(U.S Population Groups; 2007-2008 NHANES Data)**

a. Individuals were considered users if they consumed one or more food products containing cMDLf on either Day 1 or Day 2 of the survey.
b. Number of users over the number of people surveyed.
c. Percent of user when sample weights were considered.

Estimates reflect consumption of milk and milk products, including all fluid and flavored milks (excluding imitation milks), powdered, concentrated and reconstituted milks, yogurt, milk-based meal replacements, milk deserts/sauces/deserts, and cheeses. cMDLf concentrations in these products used for calculating EDI are listed below.

- 0.1 mg cMDLf/g of fluid milks including regular, filled, buttermilk and dry reconstituted (Barth and Behnke, 1997).
- 0.21 mg cMDLf/g of fluid milk (evaporated and concentrated). The average composition of milk consists of 86.6% water, 4.1% fat, 3.6% protein, 5.0% lactose, and 0.7% ash (Swaisgood, 1985). Approximately 60% water in milk is removed when milk is concentrated giving a cMDLf level of 0.1 mg/(0.134 g solid + 0.866 g x 40% water) = 0.21 mg cMDLf/g of concentrated milk.
- 0.1 mg cMDLf/g of yogurt.
- 0.1 mg cMDLf/g of flavored milk and milk drinks (fluid).
- 0.05 mg cMDLf/g of milk-based meal replacement (fluid) assuming they contain 50% milk.
- 0.75 mg cMDLf/g of dry milk powder (not reconstituted) because 0.1 mg/0.134 g dry matter in milk = 0.75 mg cMDLf /g dry milk powder.
- 0.05 mg cMDLf/g milk deserts, sauces, gravies, assuming they contain 50% milk.
- 0.75 mg/g cheese (excluding imitation cheeses). In addition to water, cheeses contain proteins and fats from milk. Some cheeses contain reduced fat. Average protein content in cheese is 25% (http://nutritiondata.self.com/). Milk protein contains 0.3% cMDLf (Barth and Behnke, 1997), giving cMDLf concentration in cheese of 0.3% cMDLf x 25% = 0.75 mg/g cheese.
- 0.075 mg cMDLf/g cheese soup assuming cheese soups contain 10% cheese.

### d) Summary of background exposure from food and infant formulas

Background intakes of cMDLf from the consumption of milk and milk-products including infant formulas (but excluding soy protein-based infant formulas) are summarized in Table 11. Because a small percentage (2.6 %) of toddlers also consumed infant formulas, the mean background exposure increased from 59 mg/d (Table 10) to 61 mg/d (Table 11). The background exposure toddlers in the 90$^{th}$ percentile also increased from 105 mg/d (Table 10) to 110 mg/d (Table 11).

**Table 11. All-User Estimated Daily Intake (EDI) of Cow's Milk-Derived Lactoferrin (cMDLf) from Background Exposure to Milk and Milk Products Including Infant Formulas**
**(U.S Population Group; 2007-2008 NHANES Data)**

| Population | $N_u/N_p$ [a, b] | Percent users[c] | Absolute EDI (mg cMDLf/d) | | Weight-based EDI (mg cMDLf/kg/d) | |
|---|---|---|---|---|---|---|
| | | | Mean | 90$^{th}$ Percentile | Mean | 90$^{th}$ Percentile |
| Infants, 0-4 mo | 126/132 | 97.3 | 102 | 150 | 17.9 | 27.0 |
| Infants, 5-11 mo | 204/221 | 93.8 | 88 | 133 | 10.0 | 14.9 |
| Toddlers, 12-35 mo | 438/446 | 98.7 | 61 | 110 | 4.9 | 9.3 |
| Children, 3-11 yr | 1311/1335 | 97.9 | 45 | 79 | 1.8 | 3.6 |
| Teens, 12-19 yr | 863/963 | 90.4 | 44 | 96 | 0.7 | 1.5 |
| Adults, 20+ yr | 4045/4628 | 88.7 | 36 | 75 | 0.5 | 1.0 |
| Total population | 6987/7725 | 90.4 | 40 | 82 | 1.0 | 2.0 |

The median value of 126 ppm (Table 9) was used in the calculation of background lactoferrin consumption from infant formula.

a. Individuals were considered users if they consumed one or more food products supplemented with cMDLf on either Day 1 or Day 2 of the survey.
b. $N_u/N_p$ denotes the number of users/number of people surveyed.
c. Percent of user when sample weights are considered.

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### c) Background exposure of cMDLf from the consumption of dietary supplements

Lactoferrin is currently marketed in dietary supplement form. Example products are listed in Table 12 with manufacturer's intake recommendations. These products are not included in the NHANES database and, therefore, not included in estimations of cumulative daily intake.

**Table 12. Concentration and Recommended Daily Intake of Cow's Milk-Derived Lactoferrin (cMDLf) in Dietary Supplements**

| Product | Manufacturer Country | cMDLf Concentration | Label Instructions | Manufacturer's Recommended Daily Intake |
|---|---|---|---|---|
| Lactoferrin | Jarrow Formulas, Canada | 250 mg/capsule | 1 capsule/d | 250 mg |
| Immune Ultra | AOR, Canada | 4820 mg/rounded scoop | 1 or 2 scoops/d | 4820-9640 mg |
| Laktoferrin with Colostrum | Allergy Research Group, USA | 100 mg/capsule | 1-4 capsules/d | 100-400 mg |
| NutriCology - Laktoferrin | Allergy Research Group | 1050 mg/3 capsules | 3 capsules/d | 1050 mg |
| High Potency Lactoferrin | Swanson Health Products, USA | 100 mg/capsule | 1-3 capsules/d | 100-300 mg |
| Lactoferrin | Symbiotics, USA | 250 mg/capsule | 1-2 capsules/d | 250-500 mg |
| Lactoferrin With Colostrum Plus | Symbiotics, USA | 29 mg/2 capsules | 4 capsules/d | 58 mg |
| Lactoferrin Caps | Life Extension, USA | 300 mg/capsule | 1 capsule/d | 300 mg |
| Laktoferrin with Colostrum | Nutricology, USA | 100 mg/capsule | 1-4 capsules/d | 100-400 mg |
| Immunity Boost, Natural Tangerine Flavor | Beveri, USA | 30 mg/stick | 1 stick/d | 30 mg |
| Lactoferrin | Immunecare, UK | 250 mg/capsule | 1-3 capsules/d | 250-750 mg |
| Note: Data retrieved from the World Wide Web on May 23, 2011 | | | | |

## C. ESTIMATED DAILY INTAKE OF cMDLf

### 1. FROM PROPOSED USES

cMDLf is proposed for use as an ingredient in yogurts, probiotic fortified unfermented milks, powdered milks (dry, not reconstituted), ice creams and sherbet, chewing gums and infant formulas at the maximum levels presented in Table 7. The maximum cMDLf level is 13 mg/100 ml in ready-to-feed formulas, 100 mg/100 g in powdered (not reconstituted) infant formulas, and 26 mg/100 ml in liquid concentrated infant formulas. Because liquid concentrated infant formulas are diluted with water at 1:1 ratio, the resulting cMDLf level is 13 mg/100 ml.

#### a) Infant Formulas

The EDIs of supplemental cMDLf through its use in infant formula are presented in Table 13. The percent of infant formula use decreases from 98.1% in 0- to 4-mo-old infants to 78.7% in 5- to 11-mo-old infants, and to 2.6% of toddlers (12- to 35-mo-old). The EDI of supplemental cMDLf for infants of 0- to 4-mo-old was 100.3 mg/day (17.5 mg/kg bw/day) and 145.5 mg/day (26.4 mg/kg bw/day) at the mean and 90$^{th}$ percentile consumption, respectively. The EDI of supplemental cMDLf for infants of 5- to 11-mo-old was 87.4 mg/day (10.1 mg/kg bw/day) and 129.0 mg/day (14.7 mg/kg bw/day) at the mean and 90$^{th}$ percentile consumption, respectively. The EDI of supplemental cMDLf for toddlers of 12- to 35-mo-old was 72.7 mg/day (7.5 mg/kg bw/day) and 112.2 mg/day (12.4 mg/kg bw/day) at the mean and 90$^{th}$ percentile consumption, respectively.

**Table 13. Estimated Daily Intake (EDI) of Cow's Milk-Derived Lactoferrin (cMDLf) from the addition of cMDLf to Cow's Milk-Based Infant Formula**

| Infants and Toddlers | Percent users | Infant formula consumption (liquid) | | Intake of added cMDLf | |
|---|---|---|---|---|---|
| | | Mean | 90th percentile | Mean | 90th percentile |
| 0-4 mo | 98.1% | 818 g/d (143 g/kg/d) | 1187 g/d (215 g/kg/d) | 100.3 mg/d (17.5 mg/kg/d) | 145.5 mg/d (26.4 mg/kg/d) |
| 5-11 mo | 78.7% | 713 g/d (82 g/kg/d) | 1052 g/d (120 g/kg/d) | 87.4 mg/d (10.1 mg/kg/d) | 129.0 mg/d (14.7 mg/kg/d) |
| 12-35 mo | 2.6% | 593 g/d (61 g/kg/d) | 915 g/d (101 g/kg/d) | 72.7 mg/d (7.5 mg/kg/d) | 112.2 mg/d (12.4 mg/kg/d) |

Notes:

1. The maximum cMDLf concentration in infant formulas is 13 mg/100 ml ready-to-feed formula, which is equivalent to 12.26 mg/100g because 100 ml ready-to-feed infant formula weighs approximately 106 g.
2. The maximum supplemental cMDLf concentration in infant formula powder is 100 mg/100 g. Powdered infant formula is reconstituted by mixing 13 g powder with 93 g water yielding 106 g formula (approximately 100 ml). The reconstituted infant formula, therefore, contains supplemental cMDLf at a concentration of approximately 13 mg/100 ml (or 12.26 mg/100g).
3. In addition to supplementation of cMDLf, infant formula also contains cMDLf as a component of the whey protein. Based on calculations derived from the US infant formulas (see Table 9), for infants 0- to 4-mo-old, cMDLf intake ranges from 82.6 -171.8 mg/d (14.4 – 30 mg/kg bw/d) for average users, and 119.9-249.3 mg/d (21.7-45.2 mg/kg bw/d) for 90th percentile heavy users; for infants 5- to 11-mo-old cMDLf intake ranges from 72-149.7 mg/d (8.3-17.2 mg/kg bw/d) for average users, and 106.3-220.9 mg/d (12.1 – 25.2 mg/kg bw/d) for 90th percentile heavy users; for toddlers of 12- to 35-mo-old, cMDLf intake ranges from 59.9-124.5 mg/d (6.2-12.8 mg/kg bw/d) for average users and 92.4-192.2 mg/d (10.2-21.2 mg/kg bw/d) for 90th percentile heavy users. Therefore, total cMDLf intake is equal to proposed supplemental cMDLf plus cMDLf originally present in whey protein.

**b) Other Food Uses**

The EDIs of supplemental cMDLf from the uses in yogurts, probiotic fortified unfermented milks, powdered milks (excluding infant formulas), ice creams and sherbets, and chewing gums are presented in Table 14. Non-fermented milk fortified with probiotics in addition to cMDLf (Table 7) is new to the U.S. consumer. Therefore, yogurt drinks were used as surrogate products for estimating the intake of cMDLf from the consumption of probiotic fortified unfermented milk.

39.5% of the total U.S. population was identified as consumers of supplemental cMDLf from the proposed uses. On an individual population basis, the highest percent users were children (54.5%). Therefore, only the all-user intakes of supplemental cMDLf have been reported to provide the most conservative estimate of intake.

Consumption of foods (excluding infant formula) supplemented with cMDLf by the total U.S. consumers resulted in an estimated mean intake of supplemental cMDLf of 142 mg/person/day or 2.7 mg/kg bw/day (Table 14). The 90$^{th}$ percentile intake of supplemental cMDLf by all users was estimated to be 273 mg/person/day or 5.8 mg/kg bw/day.

The greatest absolute intake of supplemental cMDLf was found in teenagers (12- to 19-yr-old), with EDIs of 164 mg/day on average and 336 mg/day for heavy consumers (90$^{th}$ percentile). Infants 5- to 11-mo-old had the greatest intake of supplemental cMDLf on a body weight basis, with EDIs of 7.9 mg/kg bw/day on average and 16.4 mg/kg bw/day for heavy consumers (90$^{th}$ percentile).

**Table 14. All-User Estimated Daily Intake (EDI) of Cow's Milk-Derived Lactoferrin (cMDLf) from the Addition of cMDLf to the Proposed Products Excluding Infant Formulas (not Including Background Exposure) (U.S Population Groups; 2007-2008 NHANES Data)**

| Population | $N_u/N_p$ [a, b] | Percent users[c] | Absolute EDI (mg cMDLf/d) | | Weight-based EDI (mg cMDLf/kg/d) | |
|---|---|---|---|---|---|---|
| | | | Mean | 90th Percentile | Mean | 90th Percentile |
| Infants, 0-4 mo | 2/132 | 0.8 | 5 | 15 | 0.7 | 2.2 |
| Infants, 5-11 mo | 36/221 | 19.1 | 69 | 118 | 7.9 | 16.4 |
| Toddlers, 12-35 mo | 197/446 | 51.0 | 81 | 166 | 6.4 | 12.5 |
| Children, 3-11 yr | 687/1335 | 54.5 | 125 | 260 | 4.8 | 10.0 |
| Teen, 12-19 yr | 346/963 | 37.9 | 164 | 336 | 2.7 | 5.3 |
| Adults, 20+ yr | 1643/4628 | 37.3 | 146 | 275 | 1.9 | 3.8 |
| Total population | 2911/7725 | 39.5 | 142 | 273 | 2.7 | 5.8 |

a. Individuals were considered users if they consumed one or more food products supplemented with cMDLf on either Day 1 or Day 2 of the survey.
b. Number of users over the number of people surveyed.
c. Percent of user when sample weights are considered.

Maximum supplemental cMDLf concentrations in food products for EDI calculation are.

- 1 mg cMDLf/g in yogurts and probiotic fortified unfermented milks.
- 4 mg cMDLf/g in dry milk powders (not reconstituted), excluding infant formulas.
- 2 mg cMDLf/g in ice creams and sherbets.
- 30 mg cMDLf/g in chewing gums.

## 2. CUMULATIVE EXPOSURE FROM BACKGROUND AND PROPOSED USES

Cumulative estimated daily intakes combining the background exposures (Table 11) and the exposures resulting from the supplementation of foods with cMDLf (Tables 13 and 14) are presented in Table 15. For infants 0- to 11-mo-old, cMDLf exposure approximately doubles with the proposed food uses (compare Table 11 and Table 15). For toddlers, the total cMDLf intakes are 1.7 to 1.9 times background exposure (compare Table 11 and Table 15). For children, teens and adults, the total intakes of cMDLf (from both background and supplemental exposures) are approximately 2.4 to 2.7 times (average users) and 2.7 to 3.3 times ($90^{th}$ percentile heavy consumers) background exposures (compare Table 11 and Table 15).

**Table 15. All-User Estimated Daily Intake (EDI) of Cow's Milk-Derived Lactoferrin (cMDLf) from Background Exposure and cMDLf added to Proposed Products Including Infant Formulas (U.S Population Group; 2007-2008 NHANES Data)**

| Population | $N_u/N_p$ [a, b] | Percent users[c] | Absolute EDI (mg cMDLf/d) | | Weight-based EDI (mg cMDLf/kg/d) | |
|---|---|---|---|---|---|---|
| | | | Mean | $90^{th}$ Percentile | Mean | $90^{th}$ Percentile |
| Infants, 0-4 mo | 128/132 | 98.1 | 202 | 290 | 35.2 | 51.7 |
| Infants, 5-11 mo | 206/221 | 94.8 | 173 | 273 | 19.9 | 30.9 |
| Toddlers, 12-35 mo | 438/446 | 98.7 | 105 | 207 | 8.4 | 15.7 |
| Children, 3-11 yr | 1312/1335 | 97.9 | 115 | 257 | 4.6 | 10.4 |
| Teens, 12-19 yr | 873/963 | 91.1 | 112 | 256 | 1.8 | 4.2 |
| Adults, 20+ yr | 4060/4628 | 89.0 | 98 | 233 | 1.3 | 3.1 |
| Total population | 7017/7725 | 90.7 | 103 | 243 | 2.4 | 5.3 |

The median value of 126 ppm (Table 9) was used to calculation of background lactoferrin consumption from infant formula.

a. Individuals were considered users if they consumed one or more food products supplemented with cMDLf on either Day1 or Day 2 of the survey.
b. $N_u/N_p$ denotes the number of users/number of people surveyed.
c. Percent of user when sample weights are considered.

### 3. CONCLUSIONS

Consumption data and information pertaining to the individual proposed food uses of cMDLf were used to estimate intakes of cMDLf for specific demographic groups and for the total U.S. population. This type of intake methodology is generally considered to be "worst case" as a result of several conservative assumptions made in the consumption estimates. The inherent conservatisms in the estimated intake for single-use ingredients result from: (1) the assumptions necessary for pre-market assessment of the ingredient (e.g., broadest possible food groups); and (2) the use of a maximum proposed concentration of an ingredient in food. In reality, not all proposed products will contain cMDLf, so only consumers seeking treated products will be exposed to the product at levels comparable to that calculated – a very unlikely scenario. A refinement of the estimate from actual market use would likely result in a lowering of the estimated daily intake.

In summary, the mean EDIs of cMDLf from supplementation in infant formulas are 100.3 mg/day for infants of 0- to 4-mo-old and 87.4 mg/day for infants 5- to 11-mo-old (see Table 13). After cMDLf supplementation of infant formulas, for infants 0- to11-mo-old, cMDLf intake is approximately two-times the background exposure.

Similarly, for all other population groups, the intake to cMDLf from supplementation of the proposed milk-based products increases exposure by two- to three-times background (Table 14 vs. Table 15). This results in a mean and 90$^{th}$ percentile intake by the total population of 103 and 243 mg/day (2.4 and 5.3 mg/kg/day) respectively.

## D. FOOD CODES FOR ALL PROPOSED FOOD-USES

The individual food uses based on the US Food Code system as presented in Table 16 and Table 17, which were used for estimating the daily intake of cMDLf, both background exposures and supplemental intakes. Food codes representative of each proposed use were chosen from the Food and Nutrition Database for Dietary Studies (FNDDS). In the FNDDS, the primary (usually generic) description of a given food is assigned a unique 8-digit food code. In Table 16 and Table 17, when 8-digit codes are not complete, it means that the food codes (foods) with the same numbers given are assigned with the same use level of cMDLf.

**Table 16. Foods and Cow's Milk-Derived Lactoferrin (cMDLf) Concentrations in Foods Used for Estimating Background Exposure**

| Food code[a] | Food description | Average cMDLf level (mg/g) |
|---|---|---|
| 111xxxxx | Milk, fluid (regular; filled; buttermilk; and dry reconstituted) | 0.1 |
| 112xxxxx | Milk, fluid, evaporated and condensed | 0.21 |
| 114xxxxx | Yogurt | 0.1 |
| 115xxxxx | Flavored milk and milk drinks, fluid | 0.1 |
| 116xxxxx | Milk-based meal replacements, fluid | 0.05 |
| 11710000 to 11720413<br>11740300 to 11740603 | Infant formulas, fluid, reconstituted concentrate, reconstituted dry, and ready-to-feed (milk-based formulas and therapeutic formulas excluding soy-based formulas) | 0.126[b] |
| 118xxxxx | Milk, dry, and powdered mixtures with dry milk, not reconstituted | 0.75 |
| 13xxxxxx | Milk desserts, sauces, gravies | 0.05 |
| 140xxxxx | Cheese, not specified as to type | 0.75 |
| 141xxxxx | Natural cheeses | 0.75 |
| 142xxxxx | Cottage cheeses | 0.75 |
| 143xxxxx | Cream cheeses | 0.75 |
| 144xxxxx | Processed cheeses and cheese spreads | 0.75 |
| 146xxxxx | Cheese mixtures | 0.75 |
| 147xxxxx | Cheese soups | 0.075 |

a. Food codes obtained from NHANES database
b. Average cMDLF level in Enfamil (126 ppm).

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**Table17. Maximum Cow's Milk-Derived Lactoferrin (cMDLf) Levels in Proposed Foods Used for Calculating Supplemental Intakes**

| Food code[a] | Food description | Maximum cMDLf level[b] (mg/g) |
|---|---|---|
| 114xxxxx | Yogurt | 1 |
| 11710000 to 11720413 11740300 to 11740603 | Infant formulas, fluid, reconstituted concentrate, reconstituted dry, and ready-to-feed (milk-based formulas and therapeutic formulas excluding soy-based formulas) | 0.126[c] |
| 118xxxxx | Milk, dry, and powdered mixtures with dry milk, not reconstituted | 4 |
| 13110000 to 13140900 13150000 to 13160420 13161630 | Ice cream and sherbet | 2 |
| 53112000 | Cake, ice cream and cake roll, chocolate | 0.2 |
| 53112100 | Cake, ice cream and cake roll, not chocolate | 0.2 |
| 53222020 | Cookie, cone shell, ice cream type, wafer or cake | 0.2 |
| 53222100 | Cookie, cone shell, ice cream type, brown sugar | 0.2 |
| 53430300 | Crepe, dessert type, ice cream-filled | 0.2 |
| 91611050 | Ice pop filled with ice cream, all flavor varieties | 0.2 |
| 92510730 | Fruit punch, made with soda, fruit juice, and sherbet or ice cream | 0.2 |
| 918xxxxx | Chewing gum | 30 |

a) Food codes obtained from NHANES database.
b) Maximum use levels are also presented in Table 7.
c) In NHANES database, infant formula intakes are based on ready-to-feed or after reconstitution when powdered or concentrated infant formulas are consumed. The maximum cMDLf concentration in infant formulas is 13 mg/100 ml ready-to-feed formula, which is equivalent to 12.26 mg/100g because 100 ml ready-to-feed infant formula weighs approximately 106 g. For ice cream and cherbet-containing foods, we assume they contain 10% ice creams or sherbets.

# V. SAFETY ASSESSMENT

## A. ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION OF COW'S MILK-DERIVED LACTOFERRIN (cMDLf)

### 1. INTRODUCTION

Ingested lactoferrin is handled by the body like any other dietary protein; it enters the gastrointestinal tract and can be digested by proteolytic enzymes (Kuwata et al., 1998c; Kuwata et al., 1998a). Although it is not known what fraction of ingested lactoferrin is absorbed, studies indicate that a small amount of full-length lactoferrin survives the stomach (Troost et al., 2001), passes into the small intestine, and is absorbed intact (Takeuchi et al., 2004). Because the enzymatic activities in the gastrointestinal tract increase with age (Lindberg et al., 1997), infants may have the greatest opportunity for exposure to a small amount of undigested lactoferrin. After absorption in the small intestine, lactoferrin and its fragments partition into the lymph and then are rapidly transferred to the blood. Ingested lactoferrin may also be systemically absorbed via enterohepatic cycling (Fischer et al., 2007; Talukder et al., 2003; Harada et al., 2002; Harada et al., 1999). The mechanisms that mediate absorption of lactoferrin and its fragments are unclear but may include passive and active transport across the brush border of the intestinal mucosa (Tomé and Debabbi, 1998). Once in circulation, lactoferrin can then be absorbed or phagocytosed by cells (Adlerova et al., 2008; Graham et al., 2007) or excreted in the urine (Hutchens et al., 1991; Goldman et al., 1990). It should be noted that the iron bound to absorbed lactoferrin is ultimately deposited in the bone marrow (Bennett and Kokocinski, 1979).

### 2. LACTOFERRIN FRAGMENTS

Proteolysis of full-length ingested lactoferrin generates fragments such as lactoferricin, a bactericidal protein that contains the N-region of lactoferrin (Hamosh, 1998). Lactoferricin has been detected in the gastrointestinal tract of mice, rats and humans that were administered bovine, porcine, and human lactoferrin (Kuwata et al., 1998c; Kuwata et al., 1998a; Kuwata et al., 1998b; Kuwata et al., 2001). Information is lacking regarding the susceptibility of lactoferricin and other lactoferrin fragments to further proteolysis in the gut, but one study in rats suggests that the C-lobe of lactoferrin persists longer than the N-lobe in the small intestine (Yoshise et al., 2007).

### 3. LACTOFERRIN RECEPTORS

The uptake of lactoferrin in the gastrointestinal tract and throughout the body may be mediated by its binding to lactoferrin receptors. Recognition of lactoferrin by its receptor is

somewhat species-specific, as cMDLf is reported to bind to suckling and adult rat lactoferrin receptors (Kawakami et al., 1990), but not to primate (Lönnerdal, 1994) or porcine (Gislason et al., 1993) lactoferrin receptors isolated from the gastrointestinal tract. Uptake of lactoferrin by the liver may occur via receptor-mediated endocytosis (Adlerova et al., 2008; Graham et al., 2007); however, lactoferrin is also bound by at least two non-specific binding sites on hepatocytes, the low-density lipoprotein receptor-related protein (LRP) and the major subunit of the asialoglycoprotein receptor (RHL-1) (Graham et al., 2007). The exact role of lactoferrin receptors in the in vivo distribution and processing of lactoferrin has not been comprehensively explored.

### 4. DISTRIBUTION AND CLEARANCE OF LACTOFERRIN

Any ingested lactoferrin that enters the systemic circulation is rapidly removed from the blood by distribution into the spleen, liver, kidneys, cerebrospinal fluid and the choroid plexus epithelium of the brain (Ji et al., 2006; Talukder et al., 2003; Harada et al., 1999; Bennett and Kokocinski, 1979). In liver cells, lactoferrin is targeted to lysosomes for degradation (Graham et al., 2007). In the liver, the bound iron atoms on lactoferrin are also extracted for transport to the bone marrow (Bennett and Kokocinski, 1979).

### 5. EXCRETION OF INGESTED LACTOFERRIN

Ingested lactoferrin that is absorbed into the bile (Regoeczi et al., 1994) will either be reabsorbed (Harada et al., 1999) or leave the body in the feces (Spik et al., 1982; Davidson and Lönnerdal, 1985; Davidson and Lönnerdal, 1987; Goldman et al., 1990); lactoferrin and its fragments may also appear in the urine (Spik et al., 1982; Goldman et al., 1990). Any systemically absorbed lactoferrin that is not excreted is presumed to be completely degraded at a cellular level into amino acids.

## B. PHYSIOLOGICAL EFFECTS RELEVANT TO SAFETY OF INGESTION

### 1. INTRODUCTION

cMDLf represents approximately 0.3% of the total protein in cow's milk and is present at levels much less than those of the caseins, α-lactalbumin, and β-lactoglobulin (Crittenden and Bennett, 2005). Therefore, cMDLf is normally consumed at very low quantities with the ingestion of cow-milk derived products, compared to the intake of other milk proteins in these sources. The consumption of cMDLf-supplemented infant formulas and products made from cow's milk at the proposed GRAS levels of supplementation will increase the exposure to cMDLf from background intake by two- to three-fold. The safety of this level of intake from the

perspective of hypersensitivity has been examined and a critical review of the public literature indicates that although antibodies to bovine lactoferrin exist in some patients with cow's milk allergy (CMA), there is no direct evidence indicating that cMDLf alone is the causative agent. More importantly, indirect evidence suggests that the consumption of cMDLf-supplemented products may, in fact, promote oral tolerance to cMDLf. Because additional cMDLf will be added to products already containing other milk proteins, should an individual be reactive to cow's milk, these products should be avoided to prevent the onset of symptoms associated with CMA. Furthermore, those non-milk products (chewing gum) containing cMDLF will be appropriately labeled as containing cow's milk proteins.

## 2. COW'S MILK ALLERGY, HYPERSENSITIVITY, AND ORAL TOLERANCE

CMA is often confused with lactose intolerance and broadly characterized as an inflammatory or hypersensitivity response to cow's milk proteins. CMA affects approximately 2 to 6 % of infants and 0.1 to 0.5% of adults, and for unknown reasons, a large majority (85 to 90%) of afflicted infants lose their hypersensitivity later in life (Crittenden and Bennett, 2005).

Hypersensitivity to food antigens results from the failure of the immune system to tolerate an otherwise innocuous ingested antigen (reviewed in Brandtzaeg, 2010; Mayer et al., 2001; Faria and Weiner, 2005; Mayer and Shao, 2004). Unlike "natural" or "self" tolerance, where the immune system is unresponsive to self- or auto-antigens and responsive to foreign antigens, "oral" tolerance is an active state of immunosuppression whereby unwanted responses to the gut flora and the millions of foreign antigens ingested each day are prevented.

In humans, the mechanisms that contribute to oral tolerance are largely unknown. Studies in animal models suggest that they may involve the neutralization of foreign antigens with secreted IgA antibodies and suppression of mucosal immunity either by the induction of "suppressive" T regulatory cells, or clonal anergy and clonal deletion, which are mediated by low and high doses of antigen respectively. Furthermore tolerance can be terminated by prolonged absence of exposure to a particular antigen. In newborns, the "playing field" is different. The immune system is naïve with regard to its exposure to foreign antigens, the number of circulating IgA-secreting B cells is limited, and the gut epithelium is more permeable to ingested antigens. Breast milk contains IgA, the anti-inflammatory cytokines interleukin 10 (IL-10) and transforming growth factor β (TGF-β), and small amounts of antigens that the mother is exposed to everyday (Brandtzaeg, 2010). Thus, maternal breast milk not only provides the infant with the necessary nutrients, but may also promote the development of oral tolerance by neutralizing potentially dangerous antigens with IgA, reducing inflammation with IL-10 and

TGFβ, and inducing suppressor T cells with low doses of antigen. Why one individual is hypersensitive to an otherwise innocuous food antigen is unclear, but it is currently believed to involve a combination of genetics, age, dose and timing of antigen exposure, the integrity of the gut epithelium, and properties of the antigen itself.

Hypersensitivity reactions can be categorized into 4 groups, allergic or immediate (type 1), cytotoxic (type 2), immune complex (type 3), and delayed-type (type 4) (Janeway et al., 2005). Allergic responses are the product of an aberrant humoral $CD4^+$ T helper (Th) 2 response that has skewed B cells to produce antigen-specific IgE. They are unlike cytotoxic, immune complex, and delayed-type responses because they occur in minutes, can persist for hours, and are triggered when antigen-specific IgE-bound $F_c$ receptors expressed on the surface of mast cells are cross-linked. IgE cross-linking causes the mast cells to degranulate, releasing the inflammatory mediators histamine and leukotriene, which dilate capillary venules, activate the endothelium, and increase vascular permeability. If the antigen is systemic or rapidly absorbed, histamine and leukotriene release is widespread and can result in anaphylaxis and potentially death. *In vivo* diagnostic tests used to determine if an individual has antigen-specific IgE antibodies include oral challenges, and patch and skin prick tests (SPTs), which detect antigen-specific IgE antibodies bound to dermal mast cells. *In vitro* tests include RASTs (radioallergosorbant tests), and enzyme-linked immunosorbent assays (ELISAs) (Vanto et al., 1999). Passive cutaneous anaphylaxis (PCA) is also used, but only in animals.

Cytotoxic, immune complex, and delayed-type hypersensitivity reactions develop over the course of days or wks, and can lead to diseased states. Cytotoxic reactions are triggered when antigen-specific IgM or IgG bind cognate antigens bound to or found on the surface of cells. This activates the complement cascade, which releases the inflammatory mediator C5a, and results in the recognition and lysis of the antibody/complement-bound cell by macrophages. Immune complex reactions are similar, but are triggered when antibodies encounter soluble antigen. Aggregates of the antibody and antigen then form, are deposited in tissues, and cause complement activation and Fc receptor-mediated leukocyte activation. Clinically, an individual's sensitivity to this reaction can be determined by the Arthus reaction.

Delayed-type hypersensitivity reactions are unlike allergic, cytotoxic, and immune complex reactions because they are antibody independent. These reactions primarily result from an aberrant Th1 or cell-mediated immune response. They are triggered when antigen-specific T cells re-encounter antigen presented by antigen presenting cells. The T cells are activated and produce cytokines that promote inflammation. An individual's sensitivity to delayed-type hypersensitivity reactions can be determined clinically by oral challenges and SPTs but unlike an

allergic response, the symptoms develop slowly. The prototypical delayed-type hypersensitivity test is the tuberculosis test.

Cow's milk allergy can be categorized into IgE-mediated and non-IgE-mediated responses (reviewed in Crittenden and Bennett, 2005). The mechanisms that drive non-IgE-mediated CMA are poorly defined, but are thought to include immune complex and delayed-type hypersensitivity reactions. The types of CMA, however, are not mutually exclusive, and, when exposed, CMA patients usually develop one or more cutaneous, gastrointestinal, and/or respiratory signs, including eczema, urticaria, angioderma, nausea, vomiting, diarrhea, rhinoconjunctivitis, and asthma. Anaphylactic reactions are rare. Currently, ELISAs for anti-bovine milk IgE antibodies in the serum or SPTs with whole milk are used as follow-up to discriminate between IgE-and non-IgE-mediated CMA. Also, milk avoidance is the most successful means of treating CMA, and milk oral challenges are the standard means of diagnosing CMA (Vandenplas et al., 2007). The following sections address the role of orally administered cMDLf in IgE- and non-IgE-mediated CMA (Table 18).

**a) IgE-mediated hypersensitivity**

Sixty-percent of people with CMA have IgE antibodies to cow's milk proteins and studies suggest that less than 4% these individuals have IgE antibodies to only cMDLf (Sampson, 1999; Crittenden and Bennett, 2005; Host et al., 1992; Wal et al., 1995a; Wal et al., 1995b; Natale et al., 2004; Gaudin et al., 2008). Anti-cMDLf antibodies have also been found in infants fed cMDLf-supplemented formula (1 mg/ml cMDLf) for six months and no adverse effects were reported (Brock et al., 1997; Lönnerdal and Hernell, 1994). Importantly, blinded oral challenges or SPTs with cMDLf have never been performed. In 1994, Atkinson and colleagues intraperitoneally sensitized Brown Norway rats with semi-skimmed cow's milk in the presence of the adjuvant carrageenan and elicited cow's milk protein-specific PCAs 21 days later to assess the ability of various cow's milk proteins to elicit IgE antibodies (Atkinson and Miller, 1994). Although Brown Norway rats were capable of developing IgE antibodies to a number of cow's milk proteins, lactoferrin immunoreactivity required both the removal of milk proteins from the diet prior to the sensitizing dose and an intraperitoneal booster of semi-skimmed milk seven days later. A follow-up study, designed to compare the relative allergenicity of orally or intraperitoneally administered lactoferrin in Brown Norway rats, found that orally administered lactoferrin was approximately 3000-times less potent at eliciting lactoferrin specific PCAs than intraperitoneally administered lactoferrin and occurred in only 25% of the sensitized rats (Meredith and Atkinson, 2000). Ishikado et al. (2005) performed a similar study in guinea pigs found that orally administered cMDLf was approximately 100-fold less potent at eliciting PCAs

than subcutaneously administered cMDLf. Importantly, these models have limited predictive value because they do not replicate how humans would be exposed to ingested lactoferrin. Thus, although cMDLf can induce the development of IgE antibodies, it is well tolerated and it's clinical relevance as an allergen is unknown.

In contrast, a variety of studies have also indicated that cMDLf may inhibit IgE-mediated hypersensitivity reactions: cMDLf and peptic cMDLf can reduce histamine release from IgE-cross-linked primary mast cells *in vitro* (Otani and Yamada, 1995); oral administration of cMDLf can reduce serum and antigen specific IgE levels (Kuhara and Hayasawa, 2002); oral administration of cMDLf increases the production of the anti-inflammatory cytokine IL-10 from the intestinal epithelium and the mesenteric lymph nodes (Takakura et al., 2006). Notably, IL-10 is present in breast milk and has been shown to inhibit other types of IgE-mediated hypersensitivity (Grimbaldeston et al., 2007). Thus, while ingested lactoferrin may be antigenic, it may promote oral tolerance by inhibiting mast cell degranulation and the inducing the production of IL-10.

**b) Non-IgE-mediated hypersensitivity**

The remaining 40% of people with CMA have non-IgE-mediated hypersensitivity reactions. Although the mechanisms that contribute to non-IgE-mediated hypersensitivity are poorly defined, it is currently thought that non-IgE-mediated CMA may be mediated by immune complex and/or delayed-type hypersensitivity reactions.

Studies in animal models have shown that oral administration of cMDLf can increase the development of anti-cMDLf IgA, IgM, and IgG antibodies (Debbabi et al., 1998; Miyauchi et al., 1997), and IgA and IgG immune complexes have also be found in mice fed high levels of cMDLf for prolonged periods of time (Fischer et al., 2007). IgA is primarily found in serum and mucosal secretions and not thought to mediate hypersensitivity reactions because it poorly activates complement. IgM is primarily bound to and activates B cells when it binds antigen. IgM also circulates in the blood, binds antigen, activates complement, and can induce cytotoxic reactions. IgG is found primarily in the serum and, when it encounters an antigen, activates complement. Importantly, cMDLf does not elicit inflammatory responses when injected subcutaneously or intraperitoneally in lactoferrin-sensitized mice, and can inhibit complement-mediated cytolysis *in vitro* and be safely fed to animals and humans for extended periods of time (Otani and Yamada, 1995; Fischer et al., 2007; Zimecki and Kruzel, 2000; Iigo et al., 2004; Artym and , 2003; Prgomet et al., 2007; Hellweg et al., 2008; Handl et al., 2009; Brock et al., 1997; Lönnerdal and Hernell, 1994). Thus, it is highly unlikely that consuming cMDLf-

supplemented infant formula will sensitize otherwise healthy infants to immune complex and/or cytotoxic hypersensitivity reactions later in life.

Delayed-type hypersensitivity (DTH) reactions are mediated by the cytokines produced by activated T cells. Unfortunately, the effects of cMDLf on T cell activation *in vitro* are varied, making it difficult to unambiguously define a role for cMDLF in regulating T cell activation and differentiation (Wilk et al., 2007; Actor, 2002; Rejman et al., 1992; Wong et al., 1997; Miyauchi et al., 1997; Debbabi et al., 1998; Artym and , 2003; Kobayashi et al., 2008; Hwang et al., 2007). However, cMDLf can promote the activation of antigen presenting cells *in vitro* and studies in mice have shown that cMDLf is a potent adjuvant, promoting DTH responses to ovalbumin, sheep red blood cells, and *Mycobacterium bovis* (Zimecki and Kruzel, 2000; Zimecki et al., 2002). Importantly, an experiment was performed by Zimecki and Kruzel (2000) to address the adjuvanticity of cMDLf. Mice were sensitized by subcutaneously administering sheep red blood cells in incomplete Freund's adjuvant and then subcutaneously injecting lactoferrin with the sensitizing agent, or administering it orally or intraperitoneally. Four days later the DTH responses were elicited with incomplete Freund's adjuvant, incomplete Freund's adjuvant and sheep red blood cells, or incomplete Freund's adjuvant and cMDLf. Compared to the DTH response elicited by incomplete Freund's adjuvant alone, cMDLf administered by all routes increased sheep red blood cell-specific DTH responses. Interestingly, oral and intraperitoneal administration of cMDLf inhibited the cMDLf-specific DTH response by approximately 50 percent. Sensitization with subcutaneous cMDLf increased cMDLf-specific DTH response. These results indicate that orally administered cMDLf can promoting DTH response to other antigens, consistent with it being an adjuvant, but not to itself.

**c) Conclusions**

Although it is well established that infants with CMA have anti-cMDLf IgE antibodies, there is no evidence to support a role for lactoferrin as a causative agent. Moreover, the presence of IgE antibodies to multiple milk proteins in individuals with CMA indicates that this condition is a more generalized abnormality, possibly reflecting a failure of these individuals to establish oral tolerance. Because oral tolerance to antigens like those in milk is established and maintained by a complicated network of genetic, extracellular, intracellular, and intercellular events, reactivity to lactoferrin or any other milk protein, is likely a symptom of the abnormality rather than the cause. Importantly, given that oral administration reduces an antigen's immunoreactivity, providing small amounts of cMDLf may, in fact, contribute to the development of oral tolerance.

| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| (Pierce et al., 1991) | Cloned and sequenced cow-derived lactoferrin. | Cloned cow-derived lactoferrin from an λgt 10 cDNA library constructed from salivary gland poly(A)-rich RNA and sequenced cMDLf using an Applied Biosystems 470A gas phase sequencer. Then compared the sequences of cMDLf, human, and mouse lactoferrin. | Bovine lactoferrin is 69% and 64% homologous to human and mouse lactoferrin. |
| (Host et al., 1992) | Characterized the anti-ovalbumin and cows milk protein (CMP) IgE, IgG, and IgG isotypes in infants with cow's milk protein allergy (CMPA). | Followed the levels of antibody isotypes in patients with or without CMPA for 1 yr from birth (via cord blood). CMPA diagnosis was established by the disappearance of symptoms after each of 2 dietary eliminations of cow's milk and cow's milk products, reoccurrence of identical symptoms after one challenge and exclusion of lactose intolerance and coincidental infection. SPTs and RASTs were used to determine if the CMPA was IgE-mediated or not. Measurements were made at birth, at the time of diagnosis, and before and after milk challenge at the age of 12 mo Mothers were not on a diet without milk or eggs during pregnancy or lactation. Anti-cMDLf IgE antibodies were quantified by crossed radioimmunoelectrophoresis. | IgE antibodies to all the milk proteins tested were found in both cow's milk allergy (CMA; IgE-mediated) and cow's milk intolerant (CMI; non-IgE-mediated) patients. Anti- cMDLf IgE antibodies were undetectable in cord blood; detectable in the serum in one individual with CMI at 6 mo, and developed in 5/20 patients with IgE-mediated CMPA only after being challenged with cow's milk at 12 mo of age. This study suggests that cMDLf IgE antibodies are relatively rare compared to those specific for other milk antigens and are generated after milk challenge. The relevance of these antibodies to the development of CMPA was not addressed. |
| (Rejman et al., 1992) | Determined the components of whey that are responsible for inhibiting the proliferation of peripheral blood mononuclear cells. | Peripheral blood mononuclear cells were harvested from cows and stimulated with concanavalin (ConA) in the presence or absence of increasing concentrations of the different components of whey. The degree of proliferation was quantified by $^{3}$H-thymidine incorporation. | Apolactoferrin, lactoferrin, and apotransferrin inhibited ConA-induced proliferation whereas transferrin, IgG, and serum albumin did not. This study shows that lactoferrin inhibits the proliferation of lymphocytes. |

| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| (Atkinson and Miller, 1994) | To establish an animal model for screening potentially allergenic food proteins. | Semi-skimmed cow's milk was injected intraperitoneally with carregeenan (an adjuvant) into Brown Norway rats. The rats were bled wkly and antigen-specific IgE mediated responses were assessed by passive cutaneous anaphylaxis (pCA). IgG levels were determined by ELISA. | Rats maintained on a diet containing milk and ovalbumin were relatively less sensitive to the milk challenge (1/5) than ovalbumin challenge (4/5). However, if ovalbumin and milk products were removed, the number of responders to milk challenge increased (3/5). IgG antibodies to all cow's milk proteins were detected and PCAs were the greatest for α-casein and lactoferrin. Based on these results, this study suggests that cow's milk proteins, and more specifically cMDLf, can induce the production of antigen-specific IgE antibodies when administered subcutaneously. However, positive PCAs were seen to all milk proteins and whether or not oral administration of cMDLf alone could induce the symptoms associated with cow's milk allergy in this experimental model was not determined. |
| (Lönnerdal and Hernell, 1994) | Evaluated the effect of selenium supplementation on the selenium status in formula-fed infants. | Compared the hematological status of infants (6-wk-old) that were either breast-fed or infant formula supplemented with different combinations of selenium, iron, and copper over time. For purposes of this review of supplementing infant formula with cMDLf, focused on Group A, which received 4 mg iron ($FeSO_4$); group B, 10 μg of selenium; Group C contained 4 mg of iron (1.4 mg of cMDLf and 2.6 mg of $FeSO_4$) and 10 μg of selenium. | Infants fed the different combinations of selenium, iron and cMDLf gained weight and grew at rates comparable to infants fed breast milk. Also, although there was less α2-macroglobulin in all the formula fed groups, there were no differences in the hematological indices. Thus, supplementing infant formula with selenium, iron, and cMDLf did not dramatically affect homeostasis. |

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| **Reference** | **Objective** | **Methods** | **Results** |
| (Otani and Yamada, 1995) | Is bovine lactoferrin anti-inflammatory? | Tested the ability of cMDLf to inhibit inflammatory reactions, i.e. vascular permeability, histamine release from mast cells; complement-mediated cell lysis, Arthus reaction (hypersensitivity III reaction in the skin), picryl chloride-induced contact dermatitis, and SRBC-induced delayed-type hypersensitivity. | cMDLf increased vascular permeability when injected intraperitoneally into guinea pigs but inhibited IgE-mediated histamine release from mast cells and complement-mediated cell lysis. cMDLf had no effect on the Arthus reaction or picryl chloride-induced contact dermatitis, two measurements of hypersensitivity III reactions, or SRBC-induced delayed-type hypersensitivity. The results suggest that cMDLf has anti-inflammatory activities and no effect on delayed-type hypersensitivity reactions. cMDLf was administered either intravenously or intraperitoneally. The effect of orally administering cMDLf in any of these models was not addressed. Importantly, cMDLf did inhibit IgE-mediated histamine release from mast cells suggesting that it may prevent allergic reactions in the gut when administered orally. |
| (Wal et al., 1995b) | Developed an ELISA to detect anti-β-lactoglobulin, α-lactalbumin, bovine serum albumin, lactoferrin, and the whole casein fraction IgE antibodies. | Coated plates with purified proteins derived from cow's milk and incubated the plates with serum taken from patients with CMA. Determine the presence of anti-cow's milk proteins with a secondary antibody that recognized human IgE. All patients used in this study presented with clinical symptoms of food allergy and positive RASTs to whole milk. The use of other *in vivo* diagnostic tests such as SPTs or oral challenges was not noted. | Competitive inhibition tests using purified proteins and antibodies that specifically recognized each antigen validated the specificity of ELISA. IgE antibodies that recognized all milk proteins tested were found in the sera of patients with CMA and the reactivity to each of the antigens was varied, i.e. some patients were monoreactive, some were reactive to more than one antigen, and some were reactive to all antigens. Interestingly, one patient had IgE antibodies to only cMDLf. The relevance of anti-cow's milk protein reactivity to the pathogenesis of CMA was not addressed. |

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| **Reference** | **Objective** | **Methods** | **Results** |
| (Wal et al., 1995a) | Characterized the reactivity of patients with CMA to the different components in cow's milk. | Collected sera from patients presenting with clinical symptoms of food allergy and positive RAST to whole milk. The average age of the patients was 11 mo. Importantly, the number of patients tested was not noted nor was the method used to determine reactivity and the isotype of the reactive antibodies. The different components of cow's milk were purified by cation exchange. | Thirty-five percent of the patients had IgE antibodies that recognized cMDLf and less that 4% of these patients were mono-reactive cMDLf. The exact number was not noted. Approximately 20% of the patients also had IgA antibodies to α-lactoglobulin, and lactoferrin. IgG1 and IgG4 antibodies to lactoferrin were very rare and the few patients that did have these antibodies had them at barely detectable quantities. The relevance of these antibodies to the development of CMA was not determined nor was the reactivity of the patients to the purified proteins confirmed by SPTs or oral challenge. |
| (Brock et al., 1997) | Are there antibodies to cMDLf in the serum of infants fed infant formula supplemented with cMDLf (1 mg/ml)? How does this differ from infants that are breast-fed? | Analyzed serum collected from normal infants, cow's milk intolerant (CMI) infants, breast-fed infants, formula supplemented with cMDLf (1 mg/ml) fed infants, adults, and autoimmune patients for the presence of anti-bovine and anti-human lactoferrin antibodies by ELISA. The criteria used determine cow's milk intolerance was not described nor were the isotypes of the antibodies detected in the sera. | CMI infants have anti-cMDLf antibodies whereas breast-fed infants have anti-human lactoferrin antibodies. Importantly, infants fed formula supplemented with cMDLf had high amounts of antibodies to cMDLf and also had antibodies to human lactoferrin. Antibodies to β-lactoglobulin were also detected and elevated in the CMI infants (noted as data not shown). The relevance of anti-cMDLf and anti-β-lactoglobulin antibodies to the development of CMI was not determined. |

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| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| (Miyauchi et al., 1997) | Does hydrolyzed bovine lactoferrin have immunostimulatory effects? | Stimulated splenocytes and enriched populations of T and B cells with cMDLf, cMDLf hydrosylate (H), concanavalin A (ConA), and lipopolysaccharide (LPS). The cMDLf was prepared from the whey protein fraction of bovine milk and cMDLf hydrosylate was prepared by digesting the bovine lactoferrin with Pepsin. Cellular responses were quantified by measuring the proliferative rates ($^3$H-thymidine incorporation) and antibody production. | cMDLf inhibited the proliferation of splenocytes whereas cMDLf H induced proliferation. Both responses occurred in a dose-dependent fashion and the ability of cMDLf H to induce proliferation did not require adherent cells. cMDLf and cMDLf H inhibited ConA-induced proliferation, lactoferrin inhibited PHA-induced proliferation while cMDLf H increased it, and cMDLf inhibited and LPS-induced proliferation while cMDLf H had no effect. cMDLf H induced the proliferation of enriched T and B cells and increased the production of IgM, IgG, and IgA from cultured splenocytes. The same effect was seen with Peyer's patch cells. This report suggests that cMDLf H is mitogenic to both B and T cells and while intact cMDLf may induce the cell death. cMDLf H may also induce B cells to produce immunoglobulin. It is noteworthy that the amount of proliferation observed when either cMDLf or cMDLf H was used to stimulate the cells was relatively small compared to the proliferative responses to ConA, PHA, and LPS. These results indicated that neither cMDLf nor cMDLf H are potent mitogens. |
| (Debbabi et al., 1998) | Does bovine lactoferrin affect immune homeostasis? | cMDLf (17% iron saturated; LactoBretagne) was administered to four groups of BALB/c mice (10 to 15 wk-old; n=10 for each group). Two groups received two different doses of cMDLf (0.5 mg/g or 1 mg/g body weight/d) administered through a stainless steel feeding tube for 5 consecutive d each wk for 4 wk. The control group received sterile saline using the same experimental conditions as the fed groups and the fourth group received an immunizing intramuscular injection of 0.01 mg of cMDLf in complete Freund's adjuvant and three wks later received an intramuscular injection of | Neither oral administration nor intramuscular injection of cMDLf affected the body weights of the mice. Also, no clinical abnormalities were observed over the course of the experiment. Oral administration of cMDLf increased total IgA and IgG levels in the saliva and intestinal fluid in dose-dependent fashion; IgA and IgG levels in the serum did not change. The cMDLf-fed mice also developed anti-cMDLf IgA and IgG antibodies in the intestinal fluid and serum. cMDLf stimulated splenocytes harvested from the cMDLf-fed mice produced more IgA and IgG; proliferated more when incubated either in medium alone, or restimulated with cMDLf |

| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| | | 0.01 mg of cMDLf in incomplete Freund's adjuvant. A control for the immunization with cMDLf was not performed. The mice were routinely observed for clinical signs of distress and body weights were recorded over the course of the experiment. Total and anti-cMDLf specific IgA and IgG antibodies in the saliva, intestinal fluid, serum, and media harvested from cultured Peyer's patch and splenocyte cell suspensions were quantified by ELISA. The proliferative responses of Peyer's patch and splenocyte cell suspensions to cMDLf and concanavalin A (ConA) were also determined by $^{3}$H-thymidine incorporation. | or ConA. The effect of the cMDLf immunization on antibody production has been excluded from this analysis because the experiment lacked an appropriate control. This paper suggests that orally administering cMDLf to mice generates antigen-specific B cells that are capable of producing anti-cMDLf IgA and IgG antibodies. |
| (Bhimani et al., 1999) | Can lactoferrin promote immunity to *Staphylococcus aureus*? | Human and bovine lactoferrin (85% and 97%, iron saturated, respectively) and human and bovine apo-lactoferrin (17% and 10% iron saturated, respectively) were incubated with *Staphylococcus aureus* (*S. aureus*) and their antibacterial effects were determined by zone-inhibition on agar plates. Bovine lactoferrin and bovine apo-lactoferrin was also intravenously (1 mg) or orally (20 mg/ml *ad libitum*) administered to NIH/PLCR mice one day prior to infection with *S. aureus*, and fourteen d later, the kidneys were removed, homogenized, and analyzed for the number of *S. aureus* colonies. | Apo-lactoferrin (human and bovine) directly inhibited the growth of *S. aureus* and the iron-saturated forms did not. *In vivo*, intravenous administration of bovine apo-lactoferrin prior to the *S. aureus* infection reduced both the bacterial burden in the kidneys and the percentage of infected kidneys in a dose-responsive manner. Intravenous administration of apo-lactoferrin after the infection did not affect the bacterial burden. Despite its inability to inhibit *S. aureus* growth *in vitro*, iron saturated bovine lactoferrin inhibited *S. aureus* growth *in vivo*. Importantly, oral administration of either bovine apo- or iron-saturated lactoferrin inhibited *S. aureus* growth *in vivo*. This study suggests that oral administration of bovine lactoferrin can protect mice from staphylococcal infections. |

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| **Reference** | **Objective** | **Methods** | **Results** |
| (Vanto et al., 1999) | Evaluated the value of the patch test, skin prick test (SPT), and milk-specific IgE by CAP RAST to the diagnosis of CMA. | Performed skin prick tests, patch tests, and CAP RASTs on 305 infants with suspected hypersensitivity to cow's milk. Double-blind, placebo-controlled milk challenges were preformed prior to testing determine the presence of true milk allergy. | Positive reactions were observed in 176 individuals following milk challenge (immediate reactions were observed in 100 infants and delayed reactions were observed in 76 infants). Serum milk-specific IgE levels and wheal size of the SPT were significantly greater ($p<0.05$) in patients with immediate positive reactions to milk challenge. There were no significant differences in serum milk-specific IgE or SPTs between infants with delayed positive reactions and those with a negative milk challenges. Positive SPTs were significantly associated with positive patch tests whereas positive patch tests were not significantly associated with the presence of serum milk-specific IgE. Positive patch test results were also not significantly associated with positive cow's milk challenges. However, wheal size of the SPT was also found to be discriminatory measure of immediate, delayed, and negative reactions to milk challenge. Thus, the authors propose that the SPT is the best way to determine infants with immediate-type hypersensitivity followed by anti-milk protein IgE ELISAs. |
| (Zimecki and Kruzel, 2000) | Can bovine lactoferrin act as an adjuvant? | CBA mice (8- to12-wk-old) were sensitized with sheep red blood cells (SRBC), Bacillus Calmette-Guerin (BCG), or ovalbumin (OVA) in complete or incomplete Freund's adjuvant at the base of the tail and delayed-type hypersensitivity reactions were elicited 4 d later by subcutaneously administering the sensitizing antigen into the footpad. Bovine lactoferrin (<25% iron saturated) or human lactoferrin (< 20% iron saturated) was administered intraperitoneally, subcutaneously, or orally at the time of sensitization. The intensities of the DTH responses were | When bovine lactoferrin was administered intraperitoneally, subcutaneously, or orally to mice that had been sensitized to SRBC or OVA in complete Freund's adjuvant, it did not dramatically (<1.25-fold) affect the DTH response. However, when mice were sensitized to SRBC in incomplete Freund's adjuvant, orally administered lactoferrin increased the DTH response approximately 9-fold at a low dose of immunizing SRBC ($10^6$), 2-fold at a medium dose ($10^7$ SRBC) and, <1.25-fold at a high dose ($10^7$ SRBC). Oral administration of lactoferrin also increased OVA-induced DTH responses approximately 2-fold when the mice were sensitized |

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| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| | | determined by measuring footpad swelling. The duration of lactoferrin oral administration was not commented on. | with OVA in incomplete Freund's adjuvant. When mice were sensitized to BCG in complete Freund's adjuvant, subcutaneous and oral administration of lactoferrin increased the DTH response, 2-fold and 1.25-fold respectively. Importantly, lactoferrin alone was unable to elicit a DTH response when mice were immunized with SRBC in incomplete Freund's adjuvant and either treated with lactoferrin intraperitoneally or orally at the time of immunization (Figure 7). When mice were treated with lactoferrin subcutaneously at the time of immunization, the lactoferrin elicited DTH response was approximately 1.3-fold greater than the incomplete Freund's adjuvant elicited response. This study indicates that bovine lactoferrin is an adjuvant. Furthermore, the control studies investigating the ability of lactoferrin to elicit a DTH response strongly suggests that oral administration of lactoferrin does not induce DTH. |
| (Meredith and Atkinson, 2000 - *only abstract is available only*)<br><br>000058 | To establish an animal model for screening potentially allergenic food proteins. | Increasing amounts of lactoferrin were injected intraperitoneally (IP) into Brown Norway rats in the presence of the adjuvant carrageenan (CGN). IgE-specific immunoreactivity was measured by passive cutaneous anaphylaxis (PCA) and antigen-specific IgE immunoblotting 28 d later. Increasing amounts of lactoferrin were also orally administered to Brown Norway rats by gavage twice a week with once a week IP injections of CGN for six weeks. Protein-specific immunoreactivity was determined by PCA and antigen-specific IgE immunoblotting. | IP administered lactoferrin was able to produce 50% responders at 40-50 ng. Oral administration of lactoferrin was able to produce 25% responders at 0.5 mg/kg. Assuming that an average Brown Norway rat weighs 0.3 kg, the IP dose would be 0.0016 mg/kg, which is approximately 3000-fold less that 0.5 mg/kg. Orally administered lactoferrin is much less immunogenic than IP administered lactoferrin. |

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| **Reference** | **Objective** | **Methods** | **Results** |
| (Actor, 2002) | Can cMDLf promote delayed-type hypersensitivity (DTH) responses in mice? | CBA mice (8- to 12-wk-old) were sensitized with sheep red blood cells (SRBC) by injecting them subcutaneously into the base of the tail with or without cMDLf (<25% iron saturated) in complete or incomplete Freund's adjuvant. Four d later DTH responses were elicited by subcutaneously injecting SRBC in the hind footpads. Swelling was quantified 24 h later. The effect of lactoferrin on the proliferation and cytokine production (TNFα, IL-12, IL-15, IL-10, MIP-1α, and MIP-2) of the macrophage/monocyte cell line J774A.1 was also analyzed. | Subcutaneous administration of 50 and 250 μg cMDLf with a high dose of immunizing antigen in complete Freund's adjuvant modestly increased the DTH response (approx. 2-fold). When cMDLf was administered in incomplete Freund's adjuvant with a low dose of sensitizing antigen, lactoferrin increased the DTH response (approx. 20-fold). When higher doses of immunizing antigen were used with lactoferrin and incomplete Freund's adjuvant, the DTH response increased but the effect of cMDLf was not as dramatic. Intraperitoneal injection of cMDLf alone modestly increased the number of cells recruited to the peritoneal cavity. cMDLf also increased the proliferation of J774A.1 cells and increased their production of TNFα production, decreased their production IL-10, and increased their IL-15, MIP-1α and MIP2 gene expression *in vitro*. Although the physiological significance of the *in vitro* findings are unclear, the DTH results support the notion that cMDLf is an adjuvant. |
| (Togawa et al., 2002b) | Is bovine lactoferrin anti-inflammatory? | Induced colitis in rats by injecting 2,4,6-trinitrobenzenesulfonic acid (TNBS) into the colon. Bovine lactoferrin (200 mg/kg/day; Morinaga Milk Industry) was administered directly to the stomach by gavage and inflammation was evaluated macroscopically, histologically, and biochemically. | Lactoferrin reduced the severity of TNBS-induced colitis by all parameters tested, and reduced the amount of TNFα, IL-1β, and IL-6 and increased the amount of IL-4 and IL-10 in the colonic tissue. The results of this study are similar to that published by the same group addressing the anti-inflammatory activity of bovine lactoferrin in dextran sulfate sodium-induced colitis. |

| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| (Togawa et al., 2002a) | Does bovine lactoferrin have anti-inflammatory effects in dextran sulfate sodium (DSS)-induced colitis? | Administered bovine lactoferrin (100 mg/kg or 200 mg/kg/day; Morinaga Milk Industry) though a gavage, and 3 d later induced colitis by feeding rats DSS in the drinking water. Evaluated disease severity by a disease activity index, macroscopic and histological assessment of the colon, myeloperoxidase activity, and cytokine production. Plasma and fecal concentrations of lactoferrin and the number of colonic bacteria were also determined. | Administration of bovine lactoferrin protected against DSS-induced colitis in a dose-dependent manner. Lactoferrin inhibited the DSS-induced upregulation of TNFα, IL-1β, and IL-6. It also induced IL-4 and IL-10 in the colon. Administration of bovine lactoferrin alone had no effect on any of the cytokines tested. This report indicates that lactoferrin has anti-inflammatory properties and because the development of inflammation in this model is not dependent on T or B cells, the anti-inflammatory activities of lactoferrin may directly affect on the colonic tissues. The allergenicity of bovine lactoferrin was not addressed. |
| (Zimecki et al., 2002) | Is the adjuvanticity of cMDLf dependent on a receptor that binds mannose/sugars? | CBA mice (10- to 12-wk-old) were sensitized with sheep red blood cells (SRBC) or ovalbumin (OVA) in complete or incomplete Freund's adjuvant and delayed type hypersensitivity responses were elicited with the sensitizing antigen 4 d later. cMDLf (<25% iron saturated) was administered intravenously, intraperitoneally, subcutaneously, or orally, either during sensitization or the elicitation. Mice were also treated or not with methyl-α-D-mannopyranoside. | This study contains experiments that extend the findings of Zimecki et al., 2000. The experiment in figure 4 is relevant to supplementing infant formula with cMDLf. Here the DTH-promoting effect of orally administered cMDLf when given with the sensitizing dose of OVA was inhibited by intraperitoneal administration of methyl-α-D-mannopyranoside 2 h prior to sensitization. This suggests that cMDLf acts as an adjuvant by binding a mannose-binding receptor but because the administration of cMDLf was for the 4 day prior to eliciting the DTH response the mechanisms by which this inhibition occurs is not straightforward. |

| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| (Artym and , 2003) | Can cMDLf augment the reconstitution of humoral responses in previously immunocompromised mice? | Induced lymphopenia in mice (10 to 12 wk-old) by administering cyclophosphamide (an alkylating agent) intraperitoneally. The mice were then fed cMDLf in water (~20 mg/day; <25% iron saturated; Morinaga Milk Industry) for 30 d and the effect of cMDLf on humoral responses (immunization with sheep red blood cells (SRBC) followed by the isolation of splenocytes and the determination of antibody forming cell number), reconstitution of T and B cells in the spleen and macrophages in the peritoneal cavity, and the proliferative responses of splenocytes to concanavalin A (ConA) and pokeweed mitogen (pWM) were analyzed. | cMDLf partially reconstituted the SRBC-mediated humoral response, and partially reconstituted the numbers of splenic T cells and peritoneal macrophages in cyclophosphamide treated mice. The treatment of mice with cMDLf alone led to marginal increases in the percentage of splenic T cells and B cells but did not affect the percentage of peritoneal macrophages. Although cMDLf treatment *in vivo* also did not dramatically increase the basal and mitogen-induced proliferation of *ex vivo* splenocytes harvested from treated animals. This study shows that a diet containing cMDLf may promote the homeostatic proliferation of lymphocytes, which may or may not be due to its direct affect on T and B cells *in vivo*. |
| (Takakura et al., 2003) | Can orally administered cMDLf prevent oral candiditis in immunocompromised mice? | *Candida albicans* infections were established in ICR mice (6 wk-old) by injecting prednisolone (100 mg/kg) subcutaneously 1 day prior to swabbing of their oral cavities with cotton pads soaked in a *C. albicans* cell suspension. Prednisolone (100 mg/kg) was then reinjected subcutaneously 3 d later. The severity of the infection was evaluated macroscopically, microbiologically, and histologically over the course of the infection. cMDLf was continuously administered orally via the drinking water (0.3 % ~0.5 g/kg/d) from 1 day before the infection. | The number of viable *C. albicans* increased in the mice that received orally administered cMDLf to the same degree as the controls by three d after the infection. Thereafter, the numbers of viable *C. albicans* continued to rise in the controls but declined slightly in the mice that received orally administered cMDLf. The number of tongue lesions was also slightly reduced over the course of the infection in the cMDLf -fed mice. Small reductions in the number of viable fungi and tongue lesions were also obtained when cMDLf hydrosylate was used and/or cMDLf was delivered by intragastric intubation. Lactoferricin B did not have any prophylactic effects. Although this study shows that orally administering bovine cMDLf reduces the severity of oral candidiasis, it is difficult to draw conclusions about the effect of cMDLf on different cellular aspects of this type of infection because its infectivity is dependent on a depressed immune system. The direct effects of cMDLf on prednisolone-induced suppression of the immune system was not addressed. |

| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| (Iigo et al., 2004) | Does orally administrated of cMDLf induce cytokine production in the small intestine? | 30 - 300 mg/kg/day of cMDLf (96% pure; iron content 143 ng/mg cMDLf), cMDLf hydrosylate (H; made from the cMDLf; iron content 100 ng/mg), and bovine apotransferrin (iron content 13 ng/mg; Morinaga Milk Industry) was orally administered to BALB/c mice (5 wk-old) for 24 h or for seven d and IL-18 and proinflammatory cytokine production was determined by western blot and ELISA respectively, and caspase 1 activity in the small intestine was determined by a colorimetric protease assay. Cytokine production from peritoneal macrophages treated *in vitro* with cMDLf was also analyzed by ELISA. | A single administration of cMDLf, cMDLf H, and apo-transferrin orally led to a significant and acute (within 5 hours) increase in IL-18 in the small intestine that slowly returned to baseline but did not appear to significantly affect the levels of IL-2, IL-12, IL-1β, and TNFα. In the large intestine, all three proteins increased IL-18 but the levels did not return to baseline. In the serum, cMDLf increased IL-18 acutely and its levels slowly diminished thereafter. When administered over seven d, cMDLf significantly increased IL-18 and IFNγ expression but not in a dose-responsive manner, cMDLf H and apotransferrin also increased IL-18 and IFNγ but the increase was not significant nor dose-dependent, and cMDLf H significantly reduced the amount of IL-1β and TNFα. cMDLf also significantly increased the amount of caspase 1 in the small intestine following either a single administration or seven d of continuous treatment. *In vitro*, cMDLf specifically induced IL-18 production and caspase 1 expression in peritoneal macrophages. Because IL-18 and IL-12 promote cell-mediated immunity, this study suggests that feeding mice cMDLf may induce a Th1 environment in the gut. |
| (Natale et al., 2004) | Characterized the cow's milk antigens recognized by the IgE antibodies in patients with cow's milk allergy (CMA). | Separated cow's milk proteins by 2D electrophoresis, blotted with serum obtained from patients with CMA, and developed with an anti-human IgE secondary antibody. Then isolated with immunoreactive spots and performed mass spectrometry to determine the immunoreactive protein. | Found IgE antibodies to almost all the milk proteins (except α-lactalbumin). Anti- cMDLf IgE antibodies were found in ~50% of the patients. The pathophysiological relevance of these antibodies in CMA was not addressed. |

| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| (Sfeir et al., 2004) | Does orally administered cMDLf affect immune homeostasis? | cMDLf (16.2 % iron saturated; Solarec-Soladec Recogne) was administered to BALB/c mice (4 wk; n=10 mice/group) by intramuscular injection (IM; 10 μg in complete Freund's adjuvant followed by a booster dose in incomplete Freund's adjuvant 4 wk later), intragastric gavage (IG; 0.5 ml of cMDLf (8 g/L (~ 4 mg/mouse/d))), buccal dose (BD; 0.5 ml of cMDLf (8 g/L) dropped into their mouth with the tip of an Eppendorf pipette (~ 4 mg/mouse/d)), ad libitum via the drinking water (DK; 1 or 25 g/L cMDLf solution at a level of 4 or 100 mg/mouse/day), or ad libitum as a powder in the diet (DT; 100 mg/d). Control mice received standard diet and had free access to sterile water. The amount of IgA, IgG, and IgM in the serum and intestinal secretions and secreted from cultured Peyer's patch cells and splenocytes harvested from the different groups was quantified by ELISA. *In vitro* proliferative responses and cytokine production to cMDLf and concanavalin A (ConA), and antibody production to cMDLf and lipopolysaccharide (LPS) were also determined for the Peyer's patch and splenocyte cell suspensions. | DT, BD, or IG administration of cMDLf significantly increased the amount of total IgA and IgG in the intestinal secretions 28 d post administration. At day 48 only IgG remained significantly increased in the DT, BD, and IG groups. Anti-cMDLf specific IgM, IgG, and IgA in the pooled serum and intestinal secretions increased overtime and reached a plateau at approximately 28 d post-administration. Anti-cMDLf specific IgM, IgG, and IgA also increased in the IM group but only after the booster dose. Although the analysis of $IgG_1$ and $IgG_{2a}$ subtypes in the serum and intestinal secretions lacked untreated/control mice, all methods of delivery appeared to increase anti-cMDLf $IgG_1$ antibodies. Only IG and IM of cMDLf increased $IgG_{2a}$. Splenocytes harvested from DT and BD groups proliferated more in response to cMDLf and secreted more IFNγ, IL-4, and IL-5. Splenocytes harvested from the IG and IM groups proliferated more in response to cMDLf and secreted more IL-2, IFNγ, IL-4, and IL-5. cMDLf stimulated Peyer's patch cells from the DT, BD, and IG groups produced more IL-4 and IL-5. In response to polyclonal stimulus ConA, the splenocytes harvested from the DT, BD, IG, and IM groups proliferated more and secreted more IL-4. The IG and IM group also produced more IL-2. The Peyer's patch cells from the DT, BD, and IG groups secreted more IL-4 and IL-5. Splenocytes from the IG and IM group secreted more total IgA in response to cMDLf and those from the BD, IG and IM group secreted more IgG. LPS reduced total IgA production from only the splenocytes taken from the IG group and had no effect on the IgA production by other splenocytes or on IgG production. Peyer's patch cells from the IG and IM group produced significantly |

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| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| | | | more of IgG spontaneously than the BD and control groups and cMDLf treatment increased IgA production by the cells taken from the IG group. LPS treatment also increased the production of IgG from the IG and IM group. Total IgA and IgG production from splenocytes and Peyer's patch cells from either the DT and DK were not tested. This report suggests that mice fed bovine cMDLf develop a Th2 environment in the gut that presumably generates cMDLf-specific B cells that produce IgA, IgM, and IgG antibodies specific for cMDLf. Whether or not anti-cMDLf IgA, IgM, and IgG antibodies resulted in hypersensitivity to cMDLf was not addressed nor was the development of anti-cMDLf specific IgE antibodies. Also the general health of the mice was not noted. |
| (Takakura et al., 2004) | Can oral administration of cMDLf prevent oral candidiasis in immunocompromised mice? | Oral candidiasis in mice was induced in mice (6 wk-old) by injecting prednisolone, infecting them with *Candida albicans* 1 day later, and then reinjecting prednisolone 4 d later. cMDLf was administered orally (0.5 g/kg/day continuously; Morinaga Milk Industry Co.) 1 day prior to inducing experimental oral candidiasis. The extent and severity of the infection was determined macroscopically. The cellular profile of the circulating leukocytes was determined by smears, and cervical lymphocyte cytokine production was quantified by flow cytometry. | Oral administration of cMDLf appeared to prevent the large decrease in peripheral blood leukocytes and cervical lymphocytes following the initial administration of prednisolone. After infection with *C. albicans*, cMDLf significantly inhibited number of viable fungi in the oral cavity and the tongue lesions. There were also small increases in the numbers of peripheral blood leukocytes and cervical lymph node cell and varied effects on the different cytokines produced by the cervical lymph node cells in the cMDLf -fed mice over the course of the infection but the significance of these differences is unclear because these mice all received an additional dose of prednisolone during the infection. |

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| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| (Ishikado et al., 2005) | Does liposomalization of cMDLf enhance its anti-inflammatory and immunomodulatory activities? | Prepared multi-lamellar liposomal cMDLf (Morinaga Milk Industry) and determined whether its liposomalization affected the prophylactic effect of orally administered cMDLf in $CCl_4$-induced hepatic injury and lipopolysaccharide (LPS)-induced TNFα production from peripheral blood mononuclear leukocytes (pBML). Its absorbability into the venous system, uptake into the thoracic duct, and antigenicity was also compared to cMDLf. $CCl_4$-induced hepatic injury was performed in rats by orally administering $CCL_4$, sacrificing the rats 24 h later, and quantifying the levels of glutamic pyruvate transaminase and glutamic oxaloacetate transaminase in the serum. cMDLf (300 mg/kg) was fed at 80, 56, 32 and 8 h prior to the $CCl_4$ administration. The effects of cMDLf on LPS-induced TNFα production was assessed by feeding mice cMDLf or its liposomal derivative (300 mg/kg) once a day for seven d. PBML were then harvested and stimulated with LPS *in vitro* and the amount of TNFα in the supernatants was quantified by ELISA. The absorbability of cMDLf into the blood stream was determined by infusing cMDLf or its liposomal derivative into the duodenum of rats, collecting the venous blood from the jugular vein at different time points, and determining its concentration. cMDLf uptake by the lymph was determined by using a similar method but lymph fluid was collected from the thoracic duct. The antigenicity of cMDLf was determined by feeding it5 d a wk for three wks to 6 wk-old guinea pigs (4.5 or 45 mg/body) or by injecting | Orally administered liposomal cMDLf reduced the levels of serum glutamic pyruvate transaminase and glutamic oxaloacetate transaminase in $CCL_4$-treated rats to a greater extent than cMDLf. Orally administered liposomal cMDLf also reduced the levels of TNFα secreted by LPS-stimulated PBMLs to a greater extent than cMDLf, indicating that liposomalization makes cMDLf more potent at inhibiting inflammation. However, liposomalization did not appear to affect the absorption of cMDLf into the blood stream or lymph. The antigenicity studies showed that liposomal cMDLf was just as antigenic as cMDLf when either fed orally or administered subcutaneously once a wk for three wks. It is noteworthy that the antigenicity of orally administered cMDLf was 10- to 100-fold less than if it was subcutaneously administered and 100- to 1000-fold less than ovalbumin if it was subcutaneously administered. These results indicate the liposomalization of cMDLf increases its prophylactic effects, does not increase its already weak antigenicity, and does not perturb its absorption. |

| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| | | it subcutaneously (4.5 or 45 mg/body) once a wk for three wk. The animals were then sacrificed and antibody titers were determined by passive cutaneous anaphylaxis using recombinant cMDLf or liposomal cMDLf (4.5 mg/body). | |
| (Shin et al., 2005) | Can orally administered cMDLf affect the progression of the influenza infection in mice? | 0.5 ml of a 12.5% solution of cMDLf (Morinaga Milk Industry) or bovine lactoperoxidase was orally administered to BALB/C mice (6 wk-old) once daily by gavage (~62.5 mg/mouse) 1 day prior to infection and continued to 1 day prior to sacrifice. Mice were infected intranasally with $6.6 \times 10^2$ p.f.u. of the A/PR/8/34 strain of the influenza virus. Body weights were monitored daily and the number of bronchioalveolar lavage (BAL) cells, cytokines in the BAL fluid and serum, and viral particles were quantified. | Oral administration of cMDLf did not affect the wasting associated with the influenza infection. Although the oral administration of cMDLf did not affect the number viral particles in the BAL fluid, it appeared to reduce the severity of the infection as determined by the consolidation score, a previously described means of measuring the severity of associated pneumonia. cMDLf also significantly reduced the total number infiltrating BAL cells, including macrophages and neutrophils, at 6 d after infection. |
| (Takakura et al., 2006) | Determined the effects of cMDLf on lymphocytes harvested from the intraepithelial lining of the intestines and the mesenteric lymph node. | Orally administered cMDLf or bovine serum albumin (500 mg/kg/d) by intragastric intubation for 3 d to BALB/c mice (7 to 9 wk-old). Then isolated the lymphocytes from the intestinal intraepithelium or mesenteric lymph node and determined the cellular composition and cytokine profiles by flow cytometry and ELISA, respectively. | cMDLf did not dramatically affect the distribution of $CD4^+$, $CD8^+$, and γδ T cells in either the intraepithelium or mesenteric lymph node. The absolute numbers of the different cell types was not calculated. Cultured lymphocytes harvested from the intestinal epithelium and mesenteric lymph nodes from the cMDLf-fed animals secreted more IFNγ and IL-10 spontaneously and they also secreted more IFNγ and IL-10 in response to T cell receptor crosslinking with agonistic antibodies. Although the time course for this study is relatively short and total cell suspensions were used, the results suggest that the oral administration of cMDLf induces the production of IL-10, which inhibits allergic and inflammatory events such as mast cell function, and IFNγ, which promotes cell-mediated immunity. |

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| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| (Wakabayashi et al., 2006) | Does orally administered cMDLf affect immune homeostasis? | Administered cMDLf (2.5 mg/g; 8.2% iron saturated; Morinaga Milk Industry) by gavage to mice (8-9 wk-old) and 24 h later analyzed the cellularity of the blood and spleen by flow cytometry. Also measured cytokine gene expression in the small intestine by quantitative RT-PCR. | cMDLf treatment caused a modest increase in the total numbers of circulating cells in the blood, which was accompanied by small but significant increases in the CD4, γδ, and granulocyte subtypes. In the spleen, cMDLf treatment caused a modest reduction in the total number of cells and $CD4^+$ cells. Also found the cMDLf treatment increased the NOD, IFNβ, IL-12p40 gene expression in the small intestine. Some of the effects reported in this paper are consistent with previous reports and some are not (i.e., the reduction in splenocyte cell numbers). However, the time course of cMDLf treatment is relatively short. Therefore, these effects are acute and not entirely relevant to understanding the allergenicity of bovine cMDLf in a diet. |
| (Hartog et al., 2007) | Does cMDLf have anti-inflammatory effects? | Administered cMDLf (0.1 to 25 mg/kg/day; 16% iron saturated; DMV International) by gavage and induced inflammation in the ear by injecting zymosan intradermally. The degree of inflammation was quantified by measuring the ear thickness. Also quantified the proinflammatory cytokine production (IL-6, IL-1β, and TNFα) in the ear homogenates and from LPS-induced splenocytes harvested from the differently treated animals by ELISA. To corroborate their findings they also injected zymosan into the knee joints to induce inflammation. | Low doses of cMDLf (0.1 and 1.0 mg/kg) inhibited zymosan-induced ear inflammation whereas the high doses were ineffective. The anti-inflammatory activity of cMDLf correlated with reduced amounts of TNFα, IL-6, and IL-1 in the ear. cMDLf inhibited zymosan-induced TNFα production in the splenocytes that had been harvested from the different animals and stimulated with LPS-stimulated *in vitro*. Only the highest dose of cMDLf was able to significantly inhibit the joint swelling associated with zymosan injections. These results agree with the notion that administration of cMDLf into the gut inhibits/prevents inflammation. |
| (Hwang et al., 2007) | To examine the *in vitro* effect of cMDLf on cytokine production from activated and non-activated leukocytes. | Analyzed the production of cytokines from a variety of cell types (splenocytes, macrophages, purified $CD4^+$ T cells etc.) stimulated with lipopolysaccharide (LPS) or Bacillus Calmette-Guerin (BCG) in the presence or absence of cMDLf (95% pure; | cMDLf increased TNFα production but not IL-12p40, IL-10 or IL-6 production from splenocytes; reduced IL-10, but did not affect IL-12p40, TNFα, or IL-6 produced from LPS-stimulated splenocytes. In macrophages, cMDLf reduced LPS-induced IL-10 and possibly IL-12p40 but the later was not dose |

| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| | | <20% iron saturated; PharmaReview Corporation). | dependent. cMDLf increased IL-12p40 and reduced IL-10 from BCG-stimulated bone marrow derived macrophages, and possibly increased the number IFNγ-producing BCG specific $CD4^+$ T cells in the spleens of mice immunized with BCG although the differences were very small. These results suggest that cMDLf promotes the development of Th1/cell-mediated immune responses but the majority of the effects were observed *in vitro* and very marginal. Furthermore, the effects of cMDLf on the generation of BCG-specific $CD4^+$ T cells was only observed at one time point following BCG infection, with only one immunizing dose of BCG. Other parameters such as sensitivity of the mice to the BCG infection were not analyzed or commented on. |
| (Kobayashi et al., 2008) | Does cMDLf have immunomodulatory activities on peripheral blood mononuclear cells (pBMC)? | PBMC were harvested from cats with and without feline immunodeficiency virus (FIV) and stimulated *in vitro* with concanavalin A (ConA) in the presence or absence of cMDLf (Morinaga Milk Industry). Proliferation, apoptosis, and IFNγ and IL-2 gene expression were then quantified. | cMDLf inhibited ConA-induced proliferation of PBMCs harvested from both uninfected and infected cats and prevented the upregulation of IFNγ and IL-2. cMDLf also appeared to prevent ConA-induced apoptosis, although it is difficult to conclude anything from this experiment because ConA, a known mitogen, did not induce cell cycle progression. This study suggests that cMDLf inhibits proliferation. |

| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| (Prgomet et al., 2007) | Does orally administered cMDLf affect immune homeostasis in calves? | Calves were given *post natum* 3-2-1 colostrum 2-times a day for 6 d. On day three the cMDLf fed group received colostrum supplemented with 0.54 g cMDLf (DSM Nutritional Products) at each feeding until day 6. On day 6, the calves received non-medicated milk replacer (+/- 0.16% cMDLf), water, and hay. At wk 2-3 corn was also fed. Experiment was carried out for 10 wk. The animals were weighed and sacrificed and the amount of IgG in the colostrum and blood was measured, haematocrits and cell differentials in the blood were performed, the bacterial composition in the chyme from the colon was analyzed, histological evaluation of the intestine was performed, and genes expressed in the blood leukocytes and intestine were quantified. | Calves receiving the cMDLf -containing diet gained weight normally but had larger Peyer's patches and smaller villi in the jejunum and illeum. The levels of serum IgG rose initially after treatment with cMDLf and then returned to normal. Although there were acute increases in IL-8 and IL-10 (approximately 2-fold from 2 to 4 wk after cMDLf was introduced), the trend was that the diet containing cMDLf tended to reduce IL-10, IL-8, IL-1β, and IFNγ expression in blood leukocytes. There were also varied effects of the cMDLf diet on the expression of the genes analyzed in the different parts of the intestinal tract (increased IL-10 and IL-6 in the omasum, reduced IL-6 in the illeum, and increased IFNγ in the abomasum). The cMDLf diet did not appear effect the types bacterial strains in the chyme of the colon. This study suggests cMDLf does not appear to significantly affect the development of calves. The allergenicity of cMDLf was not addressed. |
| (Wilk et al., 2007) | Does cMDLf activate antigen presenting cells? | Bone marrow macrophages were purified from mice that had been infected or not with Bacillus Calmette-Guerin (BCG) in the presence of cMDLf. BCG uptake, MHC class I, and MHC class II levels were quantified by flow cytometry. The effects of cMDLf on apoptosis and IL-12 and IL-10 secretion were quantified using the macrophage cell lines U937 and J774A.1. | cMDLf modestly increased BCG uptake and led to a greater increase in BCG-induced class II upregulation (approximately 1.5- to 2-fold). Class I upregulation was unaffected. cMDLf also modestly increased BCG-induced cell death and the IL-12:IL-10 ratio in U937 and J774A.1 cells, respectively. These results shows that cMDLf can promote the activation antigen presenting cells *in vitro* and suggests that it may be a good adjuvant. |
| (Gaudin et al., 2008) | Characterized the spectrum of anti-cow's milk antibodies in patients with cow's milk allergy (CMA). | Used a protein microarray to quantify the amount of cow's milk protein-specific IgE antibodies in serum harvested from patients that presented with various symptoms of CMA. Although the CMA patients had milk-protein specific IgE antibodies, the exact criteria used to select patients with CMA was not reported. | IgE antibodies to bovine serum albumin (BSA), cMDLf, caseins, and other milk proteins were present in the CMA serum. Reactivity to the caseins and cMDLf was the greatest, whereas reactivity to BSA was less intense, and none of the sera contained IgE antibodies that were specific to β-lactoglobulin |

| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| | | | or α-lactalbumin. Subsequent SPTs using purified components of cow's milk to corroborate the *in vitro* findings were not performed nor was the pathophysiological relevance of these antibodies to CMA addressed. |
| (Handl et al., 2009) | Does orally administered cMDLf affect immune homeostasis in beagle puppies? | Thirty-six beagle puppies were separated from their mothers three d after birth and subsequently fed a milk substitute with and without cMDLf (30, 60, and 120 mg/kg dry matter (DM)) every three h in the first wk and every four h in the second and third, four times daily in the fourth, and three times daily from wk five to wk eight. From day 32 on the dogs were also offered complete dry diet that had been sprayed with cMDLf at 0, 30, 60, and 120 mg/kg DM. After weaning at day 56, the dogs were exclusively fed the dry diet. Intestinal biopsies from the proximal duodenum and the proximal and middle colon were taken on day 14 and histologically scored for infiltrating lymphocytes in the villus and crypt structures of the duodenum, and for the presence of inflammation and disorganized architecture in the colon. Immunohistochemistry on the biopsies was also performed using antibodies that recognized IgA, IgG, IgM, CD3, CD4, and CD8 to enumerate infiltrating plasma cells and T lymphocytes. | Histologically, oral administration of cMDLf did not significantly alter the structure of the villus or crypts in the duodenum, nor did it cause any inflammation in the colon or alter the architecture of the colon. There was a small increase, although not significant, in lymphocytes and plasma cells in the mucosa of the group that received 30 and 60 mg/kg DM of cMDLf. The immunohistochemistry showed that orally administering cMDLf did not alter the numbers of $IgA^+$ or $IgM^+$ plasma cells in the duodenum and colon. $IgG^+$ cells tended to be lower (not significant) in the lamina propria at the villus bases of the duodenum and were significantly reduced in colon of the 30 and 60 mg group (30 and 25% respectively) but not in the 120 mg group. The distribution of $CD3^+$ and $CD4^+$ cells in the duodenum and colon was unaffected. Although the numbers of $CD8^+$ cells in the lamina propria were unaffected by the treatment, the number of $CD8^+$ cells in the intraepithelium of the colon were significantly increased (approximately 35 to 40%) in all the treated groups. This study suggests that although oral administration of cMDLf may cause shifts in cellular profiles in the duodenum and colon, it does cause any noticeable inflammation or alter the architecture of the colon. |

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| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| (Hellweg et al., 2008) | Does oral administration of cMDLf have immunomodulatory effects in adult beagles? | Orally administered cMDLf to adult beagles (2.5 yr-old) for 3 wk (120 mg/kg or 1800 mg/kg diet) and evaluated the cellular composition of the blood and the proliferative capacity of the circulating lymphocytes. Also analyzed the fecal concentration of anaerobes and aerobes. | Oral administration of cMDLf did not affect the weight or general health of the dogs but led to a small and significant increase in the numbers of monocytes, $CD4^+$, and $CD8^+$ cells. The peripheral blood mononuclear cells harvested from the cMDLf-fed animals also proliferated more in response to concanavalin A (ConA) at the highest dose tested. The allergenicity of cMDLf was not addressed. |
| (Kanwar et al., 2008) | Does cMDLf have anti-cancer properties? | 8-9 wk-old mice were fed a diet containing cMDLf (11.6 g/kg of 100% saturated; 93% pure; prepared from skim milk; Fonterra Co-Operative Group). Tumors were then introduced subcutaneously into the left flank of mice and, once the tumors were established, the mice were injected chemotherapeutics. Tumor size, vascularity, and anti-tumor cytotoxicity were evaluated, leukocyte infiltration and cellularity of the lymphoid tissue was determined, and the cytokine and nitrile production in the small intestine was quantified. | Oral administration of cMDLf sensitized the tumors to chemotherapeutics and prevented the reduction in lymphocytes associated with the chemotherapeutic treatment. Control mice that receive cMDLf diet alone had splenic and lamina propria hypercellularity and Th1 and Th2 cytokines in the small intestine. cMDLf was also found to bind cells in the epithelium of the small intestine, i.e. the Peyer's patch and the lamina propria. This study shows that oral administration of cMDLf sensitizes tumors to chemotherapy, which may be due to its ability to prevent the death of lymphocytes associated with the administration of chemotherapeutics. Importantly mice fed the cMDLf diet alone had hypercellularity of the spleen and lamina propria, two results that are consistent with other reports. The allergenicity of the bovine cMDLf was not addressed. |

| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| (Perez-Cano et al., 2008) | Can oral administration of cMDLf ameliorate rotavirus-induced diarrhea? | The diet of newborn Lewis rats was supplemented with either 0.3 g/kg whey protein concentrate (WPC; contained 0.92% cMDLf), 0.3 g/kg WPC plus 0.1 g/kg cMDLf, or standard infant formula by oral gavage over the course of latency (d 3 to 21). On day 8, the rats were infected intragavagally with SA-11 rotavirus. The overall health and weight was monitored as was the incidence, period, and severity of diarrhea, and fecal viral load. A variety of immune parameters were also analyzed such as the amount of IFNγ in the intestinal mucosa, serum levels of anti-SA-11 IgG antibodies, the proliferation and IFNγ production by splenocytes activated with SA-11, and the composition of lymphocytes within the intraepithelial lining of intestine. | Although the differences were not significant, WPC and the WPC supplemented with the cMDLf appeared to reduce the severity and incidence of diarrhea. There was also a corresponding increase in the fecal viral load. cMDLf supplemented-WPC with the also reduced the amount of circulating anti-SA-11 antibodies, reduced the proliferation of and IFNγ production from SA-11 restimulated splenocytes *in vitro*, increased the amount of IFNγ in the intestine, and had varied effects of the lymphocyte populations in the intestinal mucosa. The significance of these findings, however, is unclear because uninfected controls that received the different supplements were not included. Otherwise, this study shows that cMDLf has prophylactic activities and can modestly reduce rotavirus-induced diarrhea in rats. |
| (Schulmeister et al., 2008) | Analyzed the specificity of anti-human milk IgE antibodies. | Determined the presence and reactivity of anti-human milk IgE antibodies in the sera of 17 patients (infants and adults) with established CMA. CMA was diagnosed by milk challenge, positive skin prick tests (SPTs), and the presence of anti-cow's milk IgE antibodies. The presence of anti-human IgE antibodies was determined by immunblotting human milk resolved by SDS-PAGE with the sera collected from the different patients. SPTs were also performed using human and other types of milk and the mean wheal diameter was determined. | Sera from all 17 patients with CMA reacted specifically with cow's milk and human milk, although reactivity to human milk was less than cow's milk. The sera were also tested against breast milk obtained from four genetically unrelated mothers to rule out alloreactivity and in all cases tested the sera reacted with the milk obtained from the different mothers. Competitive inhibition experiments revealed that the sera from one patient that contained anti-human and anti-cow's milk IgE specifically recognized both human and cow milk antigens with minimal cross-reactivity whereas others recognized the same antigens. Subsequent analyses revealed that the some of patients' sera cross-reacted to a-lactalbumin and casein. Cross-reactivity to cMDLf was not determined. Human milk was able to elicit positive SPTs in 2 individuals known to have anti-human milk IgE antibodies. |

| Table 18. The Role of cMDLf in IgE and Non-IgE Mediated Hypersensitivity | | | |
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| **Reference** | **Objective** | **Methods** | **Results** |
| (Mossallam, 2009) | Determined the immune-potentiating effects of orally administered cMDLf in immunocompetent and immunosuppressed mice. | cMDLf was orally administered (1 mg/ml) on alternate d by a feeding syringe for 14 d (7 doses) to immunocompetent and immunosuppressed mice (swiss albino mice 4 to 6 wk-old), which received a single injection of cyclophosphamide (250 mg/kg) to induce lymphopenia. The mice were then infected with *Toxoplasma gondii* (*T. gondii*) and the mortality of the mice, and the numbers, viability, and infectivity of the tachyzoites, and the numbers of $CD4^+$ T cells in the spleen were quantified. | In uninfected immunocompetent mice, oral administration of cMDLf did not induce morbidity and led to approximately 2-fold increase in numbers of splenic $CD4^+$ T cells. In *T. gondii*-infected immunocompetent mice, oral administration of lactoferrin reduced *T. gondii*-induced morbidity from 80% to 5%, increase splenic $CD4^+$ T cells 2-fold, and significantly reduced the overall numbers and viability of the tachyzoites. cMDLf treatment in uninfected immunocompromised animals significantly increased the numbers of $CD4^+$ T cells. In *T. gondii*-infected immunocompromised mice, the oral administration of cMDLf also increased the numbers $CD4^+$ T cells, reduced morbidity, and significantly reduced the overall number and viability of tachyzoites. This study shows that cMDLf facilitates the immune response to *T. gondii*, possibly by increasing the numbers of $CD4^+$ T cells. Importantly and with regard to the allergenicity of cMDLf, cMDLf alone increased the numbers of splenic $CD4^+$ T cells but did not induce morbidity, suggesting that it was not toxic to the mice. |

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## 3. AUTOIMMUNITY

Autoimmunity results from a breakdown of self-tolerance because self-antigens, once regarded as safe, are recognized by the immune system as foreign and an immune response ensues (Janeway et al., 2005). Unfortunately, because self-antigens are ubiquitous, they are never completely eradicated. The result is a chronic inflammatory state whereby the immune system continues to respond to the never-ending supply of antigen. Although it is not known what causes autoimmunity, it is clear that genetics and environmental factors play important roles. Interestingly, evidence from animal and human studies has suggested that oral administration of antigens involved in autoimmunity may be therapeutic (Weiner et al., 2011; Mayer and Shao, 2004; Faria and Weiner, 2005).

Antibodies to human lactoferrin have been found in the serum of patients with autoimmune/chronic inflammatory diseases such as rheumatoid arthritis, systemic lupus erthyematosus, multiple sclerosis, reactive arthritis, ulcerative colitis, and autoimmune pancreatitis (Nässberger et al., 1994; Penco et al., 1998; Locht et al., 2000; Wong et al., 2009; Okazaki et al., 2000; Taniguchi et al., 2003), and, although orally administered cMDLf and human lactoferrin are well tolerated, they are immunogenic (Brock et al., 1997; Debbabi et al., 1998; Miyauchi et al., 1997; Sfeir et al., 2004). In a study conducted by Brock et al. (1997) it was found that infants fed cMDLf-supplemented formula developed anti-cMDLf antibodies, those exclusively breast-fed developed anti-human lactoferrin antibodies, and autoimmune adults had antibodies that recognize both human and cMDLf. Thus, it was proposed that bovine lactoferrin may sensitize the immune system to human lactoferrin and initiate autoimmunity. These findings have not been corroborated and, considering that lactoferrin is immunogenic and its blood levels rise during inflammation and infection, it is not surprising that individuals with autoimmune diseases have high titers of anti-human lactoferrin antibodies. The presence of antibodies that recognize cMDLf, however, is notable but could be explained by antibody cross-reactivity because of the high degree of homology between cMDLf and human lactoferrin. Unfortunately, this possibility was not ruled-out in this report and thus, the validity of these findings is questionable. In addition, it is conceivable that the anti-cMDLf antibodies found in these patients resulted from the breakdown in tolerance because autoimmunity is a breakdown in tolerance. The authors did note that antibodies to β-lactoglobulin were not present in the sera of the autoimmune patients, but this protein represents one of many present in cow's milk. Importantly, an extensive literature search has not revealed any subsequent reports showing a causal link between the consumption of cow's milk or cMDLf and the pathogenesis of these autoimmune/chronic inflammatory states.

An accumulating body of evidence has also suggested that the consumption of cow's milk during infancy is one factor in the development of type 1 diabetes (T1D). Type 1 diabetes, also known as diabetes mellitus type 1, IDDM, or juvenile diabetes, is an autoimmune disease whereby the body mistakenly destroys the β-islet cells of the pancreas resulting in insulin deficiency.

The cause of T1D is largely unknown, but as an autoimmune disorder, it is thought to depend on genetics and a variety of environmental factors. The environmental factors include dietary components, such as cow's milk and gluten consumption during infancy, viral infections, certain chemicals and drugs, and even the environment itself. Genetics is the greatest contributing factor with approximately 100% of affected individuals carrying at least one of the now 26 susceptibility loci (reviewed inKnip et al., 2005; Bluestone et al., 2010). However, among the genetically predisposed, the likelihood of developing this disease is approximately five percent and among genetically predisposed identical twins, the concordance ranges from 30 to 70% (Knip et al., 2010a; Kasper and Harrison, 2005).

In 1984 a case control study of diabetic children found that there was an inverse relationship between the duration of breastfeeding and the risk of developing type 1 diabetes (Borch-Johnsen et al., 1984). These findings were later confirmed by a study of Finnish children also diagnosed with T1D (Virtanen et al., 1991). Since then, numerous studies have been conducted, but a role for the consumption of cow's milk and/or cow's milk products by normal infants in the pathogenesis of disease is still unclear. Recent evidence from the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) suggests that the consumption of cow's milk products, as opposed to hydrolyzed cow's milk products, by genetically predisposed infants may accelerate the onset of disease (Knip et al., 2010b). There are no studies addressing a specific role for cMDLf in promoting the development of T1D in normal and/or genetically predisposed individuals.

## 4. EFFECTS ON BONE

Although human studies on the effects of cMDLf on bone structure and turnover rate are rare in the literature, *in vitro* and *in vivo* studies have been carried out which provide some insight into the potential biological mechanisms of action of this protein. Some cell culture experiments indicate an inhibitory effect of cMDLf on osteoclasts (Cornish et al., 2004; Blais et al., 2009; Yamano et al., 2010) which can actively resorb bone, plus multiple stimulatory effects of cMDLf on osteoblasts (Cornish et al., 2004; Cornish et al., 2006; Takayama and Mizumachi, 2008; Blais et al., 2009) which promote bone growth. Ovariectomized rodent models of bone growth and resorption *in vivo* have also reported positive results for orally administered cMDLf

(Guo et al., 2009; Blais et al., 2009). One published investigation of the effects of cMDLf on bone turnover in postmenopausal women reported positive results on certain serum and urine markers (Bharadwaj et al., 2009).

**a) *In vitro* effects on bone cell cultures**

In cell culture, cMDLf is reported to exert various effects on bone cells, including the dose-dependent increase of thymidine incorporation into primary cultures of human osteoblasts at concentrations ranging from 10-1000 μg/mL ($p < 0.05$) (Cornish et al., 2004), indicating a growth stimulatory effect on these bone-building cells. In this study, cMDLf was isolated from fresh milk and purified to ≥ 98% purity, as evaluated by high-performance liquid chromatography (HPLC). This cMDLf preparation, at 100 and 1000 μg/mL ($p < 0.05$), also stimulated bone nodule formation and mineralization in primary cultures of rat osteoblast-like cells during three wks in culture and also inhibited tartrate-resistant acid phosphatase expression in mouse bone marrow osteoclasts at 10-100 μg/mL ($p < 0.05$), a measure of osteoclast activity. However, cMDLf did not affect the ability of isolated mature osteoclasts to cause resorption pits at concentrations up to 100 μg/mL. This differential effect of cMDLf on newly formed versus mature osteoclasts may suggest that cMDLf is only effective in actively dividing cells; however, this finding needs to be confirmed. cMDLf at 10 and 100 μg/mL prevented serum starvation-induced apoptosis of primary rat osteoblast-like cells ($p < 0.05$), suggesting a protective effect that could occur via many potential mechanisms. In a similar study published by the same authors (Cornish et al., 2006), the effect of various cMDLf preparations on primary rat osteoblast-like cells was studied. The authors isolated cMDLf from fresh skim milk and refined it to a final purity of ~98% via HPLC; from this starting material, a deglycosylated cMDLf was produced enzymatically, as were both an apo- and holo-cMDLf. Full-length native cMDLf and deglycosylated cMDLf significantly ($p < 0.05$) and dose-dependently stimulated the growth of primary rat osteoblast-like cells, as measured via thymidine incorporation into the cells. The degree of iron-loading of cMDLf is apparently not involved in the growth stimulatory effect on these cells, as apo- and holo-cMDLfs exhibited equivalent potencies in this regard ($p < 0.05$ for a significant growth stimulatory effect by both proteins, versus control). Although full-length cMDLf has the most potent ability to stimulate growth of primary rat osteoblast-like cells in culture, the N-lobe, C-lobe, and various lactoferricin internal protein fragments also retain some of this biological activity, suggesting that cMDLf and its various protein fragments may operate via diverse pathways, possibly interacting with multiple target receptors. Additionally, this evidence suggests that proteolytic break down products of cMDLf in the gut may be able to stimulate bone growth *in vivo*. Another study by different authors (Takayama and Mizumachi, 2008) tested the effect of cMDLf (purity unstated; source: Fonterra, Auckland, New Zealand) on

MG63 human osteoblast-like cells cultured on collagen-coated plates. Addition of cMDLf at 1 μM in culture caused a significant ($p<0.01$) increase in both calcium deposition and osteocalcin production at three wks' culture, versus untreated cells. At two and three wks in culture, 1 μM cMDLf also statistically significantly ($p< 0.01$) increased alkaline phosphatase activity of the MG63 human osteoblast-like cells. However, cMDLf had no effect on the growth of these cells, via measurement of cellular metabolic activity using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent. Yamano and co-workers report that cMDLf at 1 and 10 μg/mL (purity unstated; source: Morinaga Milk Industry, Tokyo, Japan) tended to inhibit tartrate-resistant acid phosphatase in mouse preosteoclasts and osteoclasts, although only 10 μg/mL had statistically significant results in osteoclasts ($p< 0.05$) (Yamano et al., 2010). Taken together, these *in vitro* results indicate that cMDLf can stimulate the growth and matrix deposition activity of some osteoblast populations and inhibit either the function or growth of osteoclasts, which are considered beneficial effects.

**b) *In vivo* bone effects**

Ovariectomized rodent models (*e.g.*, rat, mouse) are used in the study of agents thought to exert an effect on bone loss in the context of osteopenia, which is demineralization and loss of skeletal bone due to the withdrawal of endogenous estrogen by removal of the ovaries. This model mimics the postmenopausal human state and is used to evaluate substances that may exert a protective effect that results in sparing of bone demineralization and loss over time. Guo and co-workers reported results from seventy three-month-old virgin female Sprague-Dawley rats that received either ovariectomization or sham operation and were then randomized to the following treatments (n=10/group): 1) untreated sham operated, 2) untreated ovariectomized, 3) ovariectomized plus serum albumin (BSA; protein control, 85 mg/kg body weight (bw)), 4) ovariectomized plus 0.85 mg cMDLf/kg bw, 5) ovariectomized plus 8.5 mg cMDLf/kg bw, 6) ovariectomized plus 85 mg cMDLf/kg bw, and 7) ovariectomized plus estradiol (positive control) (Guo et al., 2009). cMDLf was 95% pure and 20% iron-saturated (source: Australian Yosica Holding). cMDLf and BSA were dissolved in physiological saline and given as a single oral dose every day for three mo after ovariectomization. Sham and ovariectomized untreated rats were orally intubated with 1 mL of 0.9% physiological saline/kg body weight daily. The positive control group received 10 μg 17 β-estradiol (in corn oil)/kg body weight intraperitoneally every other day. After three mo of treatment, serum was collected for analysis and the uterus, femur, tibia and vertebrae were collected. cMDLf did not exert an effect on uterine weight at any of the three doses tested, versus either the untreated ovariectomized rats or ovariectomized rats receiving BSA, indicating that cMDLf does not demonstrate estrogenic activity *in vivo*. cMDLf, at 8.5 and 85 mg/kg/d for 3 mo, significantly prevented loss of bone

mineral density in the proximal femur and $L_{2-5}$ vertebra ($p < 0.05$), versus untreated or BSA-treated ovariectomized rats. The maximum load of the $L_5$ vertebra and the femur was also preserved, versus untreated or BSA-treated ovariectomized rats ($p < 0.05$), at these two doses of cMDLf. Serum calcium levels decreased with increasing dose of cMDLf, possibly due to the incorporation of calcium into newly forming bone. This possibility is supported by the concomitantly observed elevation in serum osteocalcin, a marker for bone formation, in the cMDLf-treated groups compared with the other treatment groups.

Blais and co-workers isolated cMDLf from fresh skimmed milk using cation exchange chromatography and purified it to > 98% by HPLC, and administered it in the diet of twelve-wk-old female C3H ovariectomized mice for 27 wks (Blais et al., 2009). One wk after surgery, the ovariectomized mice were divided into five groups (n=8/group): 1) control diet (included 140 g total milk protein/kg of diet), 2) 1 g cMDLf/kg diet (0.1%, w/w), 3) 5 g cMDLf/kg diet (0.5%), 4) 10 g cMDLf/kg diet (1.0%), and 5) 20 g cMDLf/kg diet (2.0%). Total protein was kept constant across all the diets as 140 g of milk-derived protein/kg diet. Diets were fed for 27 wks, and bone mineral density was measured at 9, 17, and 27 wks. Serum levels of cMDLf exhibited a dose-dependent increase across the groups at two mo, indicating it was at least partially absorbed intact after oral administration. Specifically, serum levels of cMDLf in mice consuming diets containing 0.1. 0.5, 1.0, and 2.0% (w/w) were approximately 0.1, 0.5, 0.9, and 1.45 μg/mL, respectively, indicating a dose-related increase in the absorption and systemic availability of cMDLf in mice. Bone mineral density in the femur and lumbar vertebrae, plus calcium content of the left femur, were measured at 27 wks; the maximum break load and yield load for the right femur were also determined. Ovariectomized mice receiving 0.5, 1.0, or 2.0% (w/w) cMDLf in the diet maintained femur bone mineral density, versus untreated ovariectomized mice (statistically significant, $P< 0.05$). The ovariectomized group receiving 2.0% cMDLf in the diet exhibited a statistically significant ($p< 0.05$) increase in femur bone mineral density versus the sham-operated controls, which suggests that cMDLf can improve bone mineral density even in non-estrogen-depleted states in mice. The 0.5, 1.0, and 2.0% cMDLf groups also maintained calcium content of the left femur, versus untreated ovariectomized controls ($p< 0.05$). However, only the 1.0 and 2.0% cMDLf groups maintained vertebral bone mineral density, versus untreated ovariectomized controls ($p< 0.05$). In contrast, the maximum break load and yield load of the right femur was maintained in all cMDLf groups, versus the untreated ovariectomized control group ($p< 0.05$). The effect of dietary cMDLf is both dose- and time-dependent, with higher doses manifesting results earlier on, such as prevented loss of whole body bone mineral density, where a protective effect was observed as early as 9 wks in the 1.0 and 2.0% cMDLf dietary groups, versus untreated overiectomized controls ($p< 0.05$). Although this study did not include a positive control (ovariectomized) group receiving estradiol, as the Guo study (2009)

did, its results were indicative of a positive effect of dietary cMDLf on maintaining various physical strength and mineral parameters of bone in ovariectomized mice. Additionally, this study was valuable in that it allows correlation of effective dietary doses (≥ 0.5% cMDLf, w/w) with the physiologically achieved serum levels of cMDLf (≥ 0.5 μg/mL) in mice.

Cornish and co-workers demonstrated a direct effect of cMDLf on new bone formation in both neonatal and adult mouse calvariae (Cornish et al., 2004). Direct injection of 4 mg cMDLf over the right hemicalvaria of adult male mice for five consecutive d caused increased new bone growth as observed via fluorochrome labeling experiments for calcein and alizarin that were carried out ten d later; specifically, new bone marrow formation was observed within the recently formed bone. A dose-dependent response was observed in the 0.4 and 4.0 mg cMDLf treatment groups, versus the control and 0.04 mg groups ($p < 0.05$), for the depth of new bone formed ten d after the last injection of cMDLf. cMDLf at 100 μg/mL also significantly increased thymidine incorporation into neonatal mouse calvariae ($p < 0.05$), indicating a growth stimulatory effect on these cells. The cMDLf used in this study was isolated and purified by the study authors from fresh milk and was reported to be ≥ 98% pure by HPLC.

The only published study documenting the bone-related effects of orally administered cMDLf in humans was carried out by Bharadwaj and co-workers, who measured the effect of ribonuclease-enriched cMDLf on bone turnover markers in postmenopausal women (Bharadwaj et al., 2009). The ribonuclease (angiogenin)-enriched cMDLf used in the study was either co-isolated from milk or separately admixed to a 1:1 ratio of angiogenin:cMDLf; therefore the purity of this preparation is estimated as ~50% cMDLf. The researchers enrolled 35 healthy, ambulatory postmenopausal women aged 45- to 60-yr old who had no menses for at least 12 mo, and randomized them into one of two groups: 1) 250 mg ribonuclease-enriched cMDLf/day (n=20; estimate 125 mg cMDLf/d) or 2) control capsules (n=15) for 180 d. All subjects received an oral calcium supplement providing 100% of the Recommended Daily Allowance (RDA) for calcium. Blood and urine samples were collected throughout the study. At the study's end, the percent change from baseline for the median levels of bone resorption markers (serum N-telopeptides, $NT_x$, and urine deoxypyridinoline, Dpd) were significantly decreased ($p < 0.001$ and $< 0.01$, respectively) and the percent change for bone formation markers (serum bone-specific alkaline phosphatase, BAP, and urine osteocalcin) was significantly increased ($p << 0.001$ and $< 0.001$, respectively), which are considered beneficial effects in postmenopausal women. However, it is not possible to attribute these activities solely to the cMDLf portion of the administered protein formulation as it also contained ~50% angiogenin. Future studies utilizing cMDLf preparations of higher purity will aid in establishing whether oral administration of this protein is useful in maintaining bone mineral density in humans.

### c) Conclusions

From analysis of published studies, it was noted that the concentrations of cMDLf that are reported to elicit biologically meaningful effects in human cells *in vitro* in the Cornish et al. (2004) (10-1000 μg/mL) and Takayama and Mizumachi (2008) (1 μM; approximately 80 μg/mL, based on 80 kDa molecular mass for cMDLf) studies are much higher than the biologically effective *in vivo* serum cMDLf levels reported in the Blais et al. (2009) mouse study (0.5, 0.9, and 1.45 μg/mL in serum), indicating that additional studies of orally administered cMDLf (dietary and gavage) are necessary. Yamano and co-workers (2010) report that 10 μg/mL of cMDLf caused a statistically significant decrease in enzyme activity of mouse osteoclasts, which is also higher than the biologically active serum levels that were reported in the Blais et al. (2009) mouse study (≥ 0.5 μg/mL). The two published *in vivo* studies on the effects of cMDLf on bone strength and mineralization in the context of estrogen-depletion have reported a positive effect at a minimum dose of 8.5 mg cMDLf/kg/d in ovariectomized rats for three mo duration (Guo et al., 2009) and at a minimum of 0.5% (w/w) in the diet of ovariectomized mice for 27 wks (Blais et al., 2009). Additionally, preliminary results on the effects of orally administered cMDLf on bone turnover markers in postmenopausal women are favorable, but future studies are needed that utilize more highly purified preparations in order to more clearly define the role of exogenously administered cMDLf on the maintenance of bone mineral density.

## C. ANIMAL TOXICOLOGY STUDIES

The safety of cMDLf produced by Morinaga Milk Industry Co. was evaluated in an acute toxicity study, a four-wk oral toxicity study, a thirteen-wk oral toxicity study, a chronic oral toxicity study and an Ames assay. Morinaga Milk Industry Lot no. MLF160996 with reported protein purity of 95.0% as determined by Kjeldahl and HPLC with iron content of 14.7 mg/100 g powder as determined by atomic absorption spectrometry was used in the thirteen-wk study and a chronic oral toxicity study. The lot number for cMDLf used was not cited. cMDLF was not acutely toxic or genotoxic in these assays. cMDLf administered by oral intubation to rats for 13 wks did not result in toxicologically significant treatment-related changes in the appearance, general condition, body weight, feed consumption, ophthalmology, hematology, blood chemistry, gross pathology, or histopathology of the animals. Thus, under the conditions of this study, the no-observed-adverse-effect level (NOAEL) of cMDLf was estimated to be in excess of 2,000 mg/kg/day. The chronic toxicity study was not available as a full report and therefore without a full data set, could not be used to derive a NOAEL.

## 1. ACUTE TOXICITY STUDY IN RATS

Nishimura (1991, as cited in GRN 77, and Yamauchi et al., 2000b) evaluated the acute toxicity of cMDLf in rats. Male and female Crj:CD(SD) SPF rats were exposed to single oral doses of 1,000 or 2,000 mg/kg cMDLf (MONL-01) or iron-saturated cMDLf (MONL-02) via stomach intubation. Control animals received vehicle alone (2,000 mg/kg water). Animals were observed for mortality, clinical signs, and any changes in general condition during a 14-day observation period following administration. Body weights were measured prior to the study and periodically throughout the observation period. After 14 d, the animals were euthanized and the organs examined macroscopically for any abnormalities.

Exposure to 1,000 and 2,000 mg/kg MONL-01 or MONL-02 resulted in no deaths or abnormal clinical signs or effects on the general condition of the animals and there were no significant differences in body weights throughout the study period in treated animals compared to controls. No abnormal gross pathological findings were observed in any organ in the cranial, thoracic, and abdominal cavities. A single oral dose of 1,000 or 2,000 mg/kg cMDLf or iron-saturated cMDLf resulted in no adverse effects or deaths. Based on these results, the lethal dose of cMDLf exceeds 2,000 mg/kg.

## 2. FOUR-WEEK ORAL TOXICITY STUDY IN RATS

The safety of cMDLf was assessed in a four-wk oral toxicity study in rats (Nishimura 1997, cited in GRN 77 as an unpublished report, and Yamauchi et al., 2000b). Four-wk-old male and female Sprague-Dawley rats were exposed by oral intubation to 200, 600, or 2,000 mg/kg/day bovine cMDLf once daily for 4 wks (28 d). Animals in the control group received water by the same route of administration. Animals were observed daily for any changes in appearance or behavior. Body weight and feed consumption were measured prior to the start of treatment and twice wkly every 3 or 4 d prior to dosing. Ophthalmology, urinalysis, hematology, and blood chemistry analyses were conducted in wk 4 or at necropsy. Animals in each group were euthanized on day 29 and observed for external abnormalities. Absolute and relative weights were determined for all organs and tissues in the cephalic, thoracic and abdominal cavities. Organs and tissues of animals in the control group and high-dose group were examined histopathologically.



There were no deaths or changes in the general condition, behavior or appearance of the animals due to administration of the test article. Body weight and feed consumption were similar in all groups throughout the study; no significant differences were observed between groups. No changes in males or females or significant differences between test and control groups were observed in urinalysis (pH, protein, ketone body, glucose, occult blood, bilirubin, urobilinogen,

color, urinary sediments, 24-hour urine volume, osmolarity, sodium, potassium, chloride, or water intake); hematology (red cell count, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, platelet count, white blood cell count, differential leukocyte count, prothrombin time, activated partial thromboplastin time, or fibrinogen), or blood chemistry parameters (GOT, GPT, LDH, ALP, total cholesterol, triglycerides, phospholipids, total bilirubin, glucose, blood urea nitrogen, creatinine, sodium, potassium, chloride, calcium, inorganic phosphorus, total protein, A/G ratio or protein fractions).

Absolute and relative body weights did not change significantly throughout the treatment period or differ significantly between groups. Unilateral and/or bilateral persistence of the hyaloid artery in the eye was observed in at least one animal in each group, including the controls group. This effect was not considered treatment-related or abnormal because it occurs naturally during the development of the eyeball and disappears with growth. Gross pathological findings observed included excoriation in the neck of 2 males and 1 female in the 200 mg/kg/day group and 1 male in the 600 mg/kg/day group, pneumatosis-like enlargement of the lung in 1 male in the 200 mg/kg/day group, a dark-red spot in the lung of 2 males in the 2,000 mg/kg/day group, and dark-red spots in the glandular stomach in 1 male and 1 female in the control group and 1 female in the 600 mg/kg/day group. Corresponding macroscopic findings were mild ulcer, mild over-inflation of the lung, and slight erosion of the stomach, respectively. One female administered 600 mg/kg/day had a fracture of the incisors. These effects are considered incidental since they were not dose-related or consistently observed among the animals. A few microscopic changes were observed in male and female animals that were not considered to be treatment-related. These were cellular infiltration and focal hemorrhage of the lung; erosion in the glandular stomach; cellular infiltration of the cecum; microgranuloma in the liver; ectopic thymus; tubular basophilia, eosinophilic body in tubular epithelium, and cellular infiltration in interstitium of the kidney; degeneration and necrosis of spermatocyte; decrease of sperm in the epididymus duct; fibrosis in the muscle layer of the esophagus; hyperplasia of ductal epithelium in the sublingual gland; and disarrangement of the retina. These changes, which were slight to mild in severity, occurred sporadically in one or two animals, and were considered incidental.

Administration of 200, 600, and 2,000 mg/kg/day cMDLf to male and female rats resulted in no deaths or treatment-related changes in body weight, feed consumption, organ weight, ophthalmology, hematology, blood chemistry, urinalysis, or gross; pathology and histology examinations. Therefore, the NOAEL of cMDLf was estimated to be in excess of 2,000 mg/kg/day.

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## 3. THIRTEEN-WEEK ORAL TOXICITY STUDY IN RATS

Repeat dose toxicity of cMDLf was investigated in a 13-wk oral toxicity study in rats (Yamauchi et al., 2000b and Nishimura 1997, cited in GRN 77 as an unpublished report). Groups of 12 male and 12 female Sprague-Dawley rats, 4 wks of age, were exposed to 200, 600, and 2,000 mg/kg/day cMDLf by oral intubation once daily for 13 wks. Control animals received vehicle (water) alone. Animals were examined daily for changes in appearance, nutrition condition, or behavior. Body weight and feed consumption measurements were taken prior to the start of treatment and twice wkly every 3 or 4 d prior to dosing. At the final wk of the study, ophthalmology examination and fasting urinalysis (4-hr urine sample; one day's water consumption was calculated at the same time), fasting (16-hour) hematology (mean corpuscular volume, red cell count, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte ratio, platelet count, white blood cell count, differential leukocyte count, prothrombin time, activated partial thromboplastin time, or fibrinogen), and fasting (16-hour) blood chemistry determinations (glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), total cholesterol, triglycerides, phospholipids, total bilirubin, glucose, blood urea, nitrogen, creatinine, sodium, potassium, chloride, calcium, inorganic phosphorus, total protein, albumin:globulin (A/G) ratio or protein fractions) were completed (urinalysis was also done at wk 6). Animals were euthanized after 91 d and observed for external abnormalities. Absolute and relative weights were determined for all organs and tissues in the cephalic, thoracic, and abdominal cavities. Histopathological examinations were conducted of all organs and tissues in the control and high-dose animals, the pancreas of males at all dose levels, and in any animal that died or exhibited macroscopic lesions.

There were two deaths during the treatment period. One male in the 200 mg/kg/day group died at wk 10 without any overt clinical signs of disease. Examination revealed perforation in the esophagus and hydrothorax with feed. The death was attributed to an error in intubation. One female in the 2,000 mg/kg/day group exhibited swelling of the subcutis in cervical, axillary and inguinal regions at wk 12 and died at wk 13. At necropsy, enlargement of the lymph nodes, thymus, spleen, and liver and white spots in the kidney were observed. Upon histologic examination, malignant lymphoma and tumor cells in the brain, harderian gland, heart, lung, pituitary, adrenal, ileum, cecum, ovary, uterus and bone marrow were observed. Slight extramedullary hematopoiesis was seen in the adrenal gland. Death of the animal was attributed to the presence of malignant lymphoma not related to test article administration.



No abnormal clinical signs due to administration of cMDLf were seen in surviving animals. However, a few clinical signs deemed incidental because of their sporadic occurrence

were observed. These were subcutaneous mass in the axillary mass accompanied by hemorrhage and paleness of skin in 1 male in the control group, fracture of incisors in 1 female in the control group, and excoriation in the neck in 1 female in the 200 and 600 mg/kg/day groups and 2 females in the 2,000 mg/kg/day group. One female in the 2,000 mg/kg/day group had decreased spontaneous movement and oligopnea. Perforation in the esophagus, hydrothorax, and dark-reddening of the lung was observed at necropsy suggesting that the effects were due to an error in administering the compound.

No significant differences were observed in body weight or feed consumption between groups during the treatment period. No ophthalmologic abnormalities were observed in any animal. Hematology and blood chemistry determinations did not indicate any statistically significantly differences in any of the parameters between the control and test groups. No test article related statistically significant changes were seen in the urinalysis parameters at wks 6 or 13 with the exception of lowered urinary pH in males and females of the 2000 mg/kg group. At wk 13, significant increases in urine volume and daily excretion of sodium, potassium, and chloride in males from the low-dose group only were noted. These changes, however, are not considered test related because they occurred in only one gender and were not dose-related. The change in urinary pH, while possibly related to test article administration, is not thought to be of toxicological significance because there were no significant changes in any other urinary parameters such as volume and content of electrolytes. In addition, there were no corresponding adverse findings in the kidney or blood chemistry values.

No changes in absolute or relative organ weights were observed in males or females administered 200 or 600 mg/kg/day. At the highest dose administered, significant decreases in absolute and relative thyroid weights compared to controls were observed in females only ($p \leq 0.05$). These changes were not considered to be test article related since they were slight, observed in only one sex and no corresponding findings of toxicity were observed upon histopathological examination.

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Gross pathology findings in a few animals and histopathologic changes in several organs were noted but these findings were not consistently observed among animals or were also noted in control animals. No histopathological findings were considered to be treatment related, but were judged incidental in view of their occurrence and the nature of the lesions. Slight or mild islet fibrosis of islet acinar cells was observed in 3/12 males in the control group and 7/12, 6/12 and 6/12 males each in the 200, 600, and 2,000 mg/kg/day groups, respectively. This finding was not seen in females. Although the incidence and severity of the finding in each treated group was higher than that of the controls, these findings were not considered to be treatment related. No morphological differences in the fibrosis of islets between the control and treated groups were

observed and the distribution of lesions in animals in the treated groups, were limited to the same small section of tissue as in the control animals. In addition, an age-dependent increase in the incidence of non-neoplastic pancreatic islet lesions, particularly fibrosis and hyperplasia, in male Sprague-Dawley rats has been noted in the literature (Imaoka et al., 2007). Incidence of fibrosis in pancreatic islets of male Sprague-Dawley rats fed standard diet at 8, 12, 18, and 26 wks of age was 0/20, 3/20, 14/20 and 18/20, respectively. In females, the incidence was 0/20, 1/20, 0/20 and 4/20 for the respective time points.

cMDLf administered by oral intubation to rats for 13 wks did not result in toxicologically significant treatment-related changes in the appearance, general condition, body weight, food consumption, ophthalmology, hematology, blood chemistry, gross pathology, or histopathology of the animals. Thus, under the conditions of this study, the NOAEL of cMDLf was estimated to be in excess of 2,000 mg/kg/day.

## 4. CHRONIC ORAL TOXICITY STUDY IN RATS

In a research communication (Tamano et al., 2008), results of long-term feeding studies of cMDLf in two experiments were reported. Male and female F344/DuCrj (F344) rats, 5 wks of age were purchased. In Experiment 1, starting at 6-wks-of-age, groups of 15 male rats were given a diet containing 0% (control) or 0.2% cMDLf for 40 wks. Animals were observed for general condition every day and weighed once wkly for the initial 4 wks and once every 4 wks thereafter. Feed consumption by cage was performed at the same time as body weight measurement. Test material intake was calculated. At the end of treatment, all animals were fasted overnight and in the morning, whole blood samples were collected by determination of aspirate aminotransferase, alanine aminotransferase, gamma-glutamyltranspeptidase, alkaline phosphatase, blood urea nitrogen, creatinine, glucose, total cholesterol, triglyceride, total protein, albumin and serum iron. Gross inspections for any lesions were made at necropsy and liver, kidneys and spleen were weighed and relative organ weights calculated. In Experiment 2, starting at 17 (males) or 11 (females) wks of age, groups of 25 rats (groups 1 and 5) and 10 rats (groups 2, 3,4) of each sex were given diet with 0, 0.02, 0.2, 2.0, and 5.0% cMDLf (groups 1-5, respectively) for 60 wks in males and 65 wks in females. The animals were observed for general condition daily and weighed 8 times during the study. Measurement of feed consumption and water intake by cage were performed once every 2 wks for the first 16 wks and once every 4 wks thereafter. Test material intake was calculated for each group. Gross examination was done at necropsy. Liver, kidney, spleen, adrenals and pituitary were weighed for each animal. Samples of these organs, thymus, lungs, salivary glands, esophagus, stomach, duodenum, jejunum ileum, cecum, pancreas, urinary bladder, testes, prostate, seminal vesicle, ovaries, uterus, vagina, spinal cord and grossly visible lesions were fixed and processed for histopathological examination.

In Experiment 1, no adverse treatment-related clinical signs, effects on body weight, or macroscopic changes were reported (data not shown). Slight but significantly decreased relative (but not absolute) liver weights were reported (data not shown). Selected blood biochemistry data were reported: AST, ALT, ALP, BUN and TG were significantly lower in the 0.2% group compared to control. No treatment-related histopathological lesions were observed (data not shown).

In Experiment 2, no adverse clinical signs or deaths were reported during the study period. No adverse effects on body weight, feed or water consumption was reported (data not shown). There were no significant treatment-related adverse effects on final body weight, organ weight, gross or histopathology reported (data not shown).

## 5. GENOTOXICITY

The genotoxic potential of cMDLf was examined in the reverse mutation assay (Yamauchi et al., 2000b). The assay was performed using *Salmonella typhimurium* strains TA100, TA1535, TA98, and TA1537 and *Escherichia coli* strain WP2uvrA, with and without metabolic activation. Metabolic activation was provided by an Aroclor-induced, rat liver microsome fraction (S9 mix). The tester strains were exposed to cMDLf via the preincubation method. cMDLf was tested at six concentrations: 160, 320, 630, 1,250, 2,500, and 5,000 μg/plate, based on the results of a dose range finding test. Physiological saline was used as the vehicle control. In the absence of metabolic activation, the positive controls were 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide for tester strains TA100, TA98, and WP2uvrA; sodium azide for tester strain TA1535, and 9-aminoacridine for tester strain TA1537. In the presence of metabolic activation, the positive controls were benzo(a)pyrene for tester strains TAl00, TA98, and TA1537 and 2-aminoanthracene for tester strains TAl535 and WP2uvrA. cMDLf was evaluated in duplicate for each concentration in the presence or absence of metabolic activation.

No precipitation or crystallization was observed at any cMDLf concentration. The positive controls for the tester strains yielded the expected number of revertants/plate of at least a 2-fold increase in the number of revertants relative to the vehicle control. For the test article to be classified as producing a positive result, there had to be a proportional increase in the number of revertant colonies relative to the increase in the concentration of cMDLf and the ratio of the number of revertant colonies to that of the control group had to be at least 2.0. For all concentrations of cMDLf in all tester strains, with or without metabolic activation, the ratio of the number of revertant colonies to that of the control group was 1.4 or lower. A second assay produced similar results.

cMDLf did not cause a positive response with any of the tester strains in the presence or absence of S9 activation at concentrations up to 5,000 μg/plate. Thus, under the conditions of this study, cMDLf was not found to be genotoxic in the *Salmonella typhimurium* reverse mutation assay or *Escherichia coli* mammalian microsome reverse mutation assay.

## D. HUMAN STUDIES OF cMDLf

### 1. SUMMARY

Numerous studies have been published on the effects of oral ingestion of cMDLf in adults, children (Tables 19 and 20), and infants (Table 21). Dose ranges in adults and children from 100 mg/day to 3.6 g/day and durations ranging from one wk to one yr have been shown to be safe and well tolerated under the conditions of administration in these studies. Studies carried out in both healthy and health-compromised adult and child subjects reported no treatment related side effects attributed to oral ingestion of cMDLf. Studies of oral ingestion of cMDLf in health-compromised individuals and/or ingestion in conjunction with other medications, such as antibiotics, were more likely to report adverse effects across all groups, but these effects were not attributed to cMDLf ingestion, with the exception of a study citing a dose-related increase in adverse effects in chronic hepatitis C patients (high dose of cMDLf of 7.2 g/day). No adverse effects of cMDLf administration were noted on clinical chemistry and hematology parameters from clinical trials that reported these endpoints.

Administration of cMDLf at 200 mg/day for up to 90 d in pregnant women was well tolerated (highest dose tested in this target population). In term and preterm infants, exposure to cMDLf from infant formulas has been studied using concentrations ranging from 10 mg/100 mL (0.01%) to 285 mg/100 mL (0.285%), comprising durations ranging from two wks to one yr. Resulting intake of cMDLf is up to 150 mg/kg/day. No treatment related adverse effects have been reported.

Studies have reported investigation of the effects of oral supplementation of cMDLf on various microbes and viruses in adults and children. Importantly, in no instance was cMDLf reported to exacerbate existing infections or their associated clinical symptoms. Additionally, some favorable effects of cMDLf on bacterial or protozoal infections have been reported in adults and children and a possible beneficial effect on gut microflora has been demonstrated in infants. Published studies also suggest that oral ingestion of cMDLf may be beneficial for maintaining iron homeostasis in infants and women.

## 2. CLINICAL STUDIES OF cMDLf IN RELATIVELY HEALTHY ADULTS

No report of adverse effects of cMDLf ingestion was noted in healthy adults given 2 g/day cMDLf for 4 wks (Yamauchi et al., 1998). The study enrolled ten healthy male volunteers aged 31-55. The methods did not specifically mention recording adverse effects; however, no adverse findings related to tolerance were reported.

Ono and co-workers investigated the effect of cMDLf in healthy, overweight Japanese adults with a body mass index (BMI) > 25 kg/m$^2$ and a visceral fat area > 100 cm$^2$ (Ono et al., 2010). Thirty subjects were enrolled; two did not meet inclusion criteria. The remaining 28 subjects were randomized to either lactose tablets (control; $n$=14) or enteric-coated cMDLf tablets (300 mg cMDLf/d; $n$=14) for eight wks, following a two-wk run-in period. No adverse events were observed; cMDLf did not adversely affect systolic or diastolic blood pressure, pulse rate, or blood lipid parameters, versus control. Favorable effects on weight and BMI were also observed in the cMDLf group.

Bharadwaj and co-workers administered a unique, ribonuclease (angiogenin)-enriched preparation of cMDLf to postmenopausal women (Bharadwaj et al., 2010). Thirty-eight healthy postmenopausal women aged 45- to 60-yr old with no menses for at least 12 mo were enrolled. Three were excluded based on their history of treatment for bone health or hypothyroidism. The remaining women were randomized to receive either 250 mg of the cMDLf preparation (n=20; estimate 125 mg actual cMDLf, based on reported purity of ~50%)/day or placebo capsules (n=15) for 180 d. One subject in the treatment group was dropped for non-compliance; three from the control group dropped out as well. All subjects had > 95% compliance with supplements. The body weight and blood pressures of all the subjects were maintained within ± 3% of their baseline values. No adverse events were reported by the authors to have occurred during the study or the 3-month follow-up period.

Kozu et al. (2009) reported effects of cMDLf ingestion, enrolling two hundred fifteen patients aged 40-75 yr who had adenomatous colorectal polyps. Patients were randomized and assigned to placebo (n=35) or 1.5 g/d (n=37) or 3.0 g/d (n=34) cMDLf (supplied by Morinaga Milk Industry; 10-20% iron-saturated) for twelve mo. Two subjects were excluded due to not having a target polyp (placebo group) and use of statins (3.0 g cMDLf group). Two placebo group subjects withdrew from the study for reasons not stated. Two subjects were excluded from the full analysis set due to use of non-steroidal anti-inflammatory drugs. Two subjects in the 3.0 g group were found to have lung and liver metastases; both had a history of colon cancer. A mild increase in alkaline phosphatase was observed in one subject in the 1.5 g group and a moderate increase in total bilirubin was observed in one subject in the 3.0 g group. Both biochemical

parameters returned to normal at the end of the study and the authors reported that no other serious adverse events occurred during the study.

## 3. CLINICAL STUDIES OF cMDLf IN ADULTS AND CHILDREN

Studies have reported investigation of the effects of oral supplementation of cMDLf to evaluate activity of cMDLf on infection endpoints, many studies include relevant safety information. Importantly, in no instance was cMDLf reported to exacerbate existing infections or their associated clinical symptoms. Additionally, some favorable effects of cMDLf on bacterial (Di Mario et al., 2003; Di Mario et al., 2006; Tursi et al., 2007; Kondo et al., 2008; Mueller et al., 2011) or protozoal (Ochoa et al., 2008) infections have been reported, especially when cMDLf was given in conjunction with an antibiotic regimen (Di Mario et al., 2003; Di Mario et al., 2006; Tursi et al., 2007). In some cases, improvements in clinical symptoms or biochemical parameters have been observed (Yamauchi et al., 2000a; Iwasa et al., 2002; Okada et al., 2002; Konishi et al., 2006; Ishikado et al., 2010). Results on clinical symptom improvement were mixed in studies administering cMDLf to patients with chronic hepatitis C although the high doses of cMDLf that were administered were generally well tolerated by this population (Iwasa et al., 2002; Okada et al., 2002; Ishii et al., 2003; Konishi et al., 2006; Ueno et al., 2006).

Okuda and co-workers investigated the effects of cMDLf alone on *H. pylori* colonization in adults and children (Okuda et al., 2005). They enrolled 25 healthy children and 34 healthy adults having *H. pylori* infection either without or with minimal upper gastrointestinal symptoms and who were not currently being treated. Infection was diagnosed by positive reactions in both the $^{13}$C-urea breath test and serum- or urine-based enzyme-linked immunosorbent assay (ELISA), although it was not stated what value was considered the cutoff for a "positive" result in the breath test, and baseline values varied widely across all groups. Subjects who were milk intolerant were excluded and the treatment groups received either 1) two 100 mg cMDLf tablets/twice a day (400 mg/d total) (n=17, adults), 2) placebo tablets (n=17, adults), 3) two 100 mg cMDLf tablets/twice a day (400 mg/d total) (n=14, children), or 4) placebo tablets (n=11, children) for 12 wks. cMDLf was supplied by Morinaga Milk Industry. After 12 wks supplementation, 10 of 31 combined subjects (6 adults, 4 children) receiving cMDLf treatment had a > 50% decrease in their urea breath test value, versus baseline (no statistics). In most responders receiving cMDLf, the urea breath test values returned to baseline levels by 4 wks after the end of the study. The methods did not specify recording of adverse effects, however, there was no report of treatment-related adverse effects.

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Four studies investigated the effect of adding cMDLf to either triple or quadruple antibiotic therapy for treating *Helicobacter pylori* infection in adults. Di Mario and co-workers

carried out two large studies in *H. pylori*-positive patients who received triple antibiotic therapy either with or without simultaneous oral cMDLf supplementation (Di Mario et al., 2003; Di Mario et al., 2006). The first study enrolled 150 patients having dyspeptic symptoms, gastritis and peptic ulcer disease and randomized patients to receive either 1) Group A: rabeprazole (20 mg 2×/d), clarithromycin (500 mg 2×/d), and tinidazole (500 mg 2×/d), plus cMDLf (200 mg 2×/d; 400 mg/d total) for 7 d (n=51), 2) Group B: the triple antibiotic regimen alone for 7 d (n=52), or 3) Group C: the triple antibiotic regimen alone for 10 d (n=47) (Di Mario et al., 2003). Subjects were considered noncompliant if < 90% of study medication was taken. Major side effects leading to treatment discontinuation were observed in six patients: two patients in Group A (dizziness, headache, fatigue, nausea), one patient in Group B (nausea, hypotension and taste disturbance) and three patients in Group C (fatigue, nausea and diarrhea). All but 27 patients had a negative $^{13}$C-urea breath test or *Helicobacter pylori* stool antigen test by two mo after the end of therapy. The eradication rates in the groups, based on intent-to-treat analysis, were as follows: Group A, 92.2% (p=0.01, versus Groups B and C); Group B, 71.2%; Group C, 70.2%. The second study enrolled 402 patients who were randomized to receive either 1) Group A: esomeprazole (20 mg 2×/d), clarithromycin (500 mg 2×/d), and tinidazole (500 mg 2×/d) for 7 d (n=136), 2) Group B: cMDLf (200 mg 2×/d; 400 mg/d total) for 7 d, followed by the triple antibiotic regimen for 7 d (n=132), or 3) Group C: concurrent treatment with both the triple antibiotic regimen plus cMDLf for 7 d (n=134) (Di Mario et al., 2006). Of the 402 patients, 389 completed the study. Six patients were discontinued due to side effects, one patient in Group B died because of a street incident and six patients were lost to follow up. The incidence of side effects was 9.5% in Group A, 9% in Group B and 8.2% in Group C. The *H. pylori* eradication rate was significantly higher in group 3 versus groups 1 and 2 (p=0.01, intent-to-treat analysis). These two studies suggest that concurrent administration of cMDLf at 200 mg twice/day (400 mg total) may have favorable effects in combination with standard triple antibiotic therapy for the elimination of *H. pylori*.

Zullo and co-workers also investigated the effect of adding cMDLf to triple antibiotic therapy for *H. pylori* infection in 133 patients with dyspepsia who underwent endoscopy and a rapid urease test for positive diagnosis (Zullo et al., 2005). Patients were randomized to receive 1) esomeprazole (20 mg 2×/d), clarithromycin (500 mg 2×/d), amoxicillin (1 g 2×/d) for 7 d (n=68) or 2) the antibiotic regimen plus cMDLf (200 mg 2×/d; 400 mg total) for 7 d (n=65). Bacterial eradication was checked at 4-6 wks after commencement of treatment by using a $^{13}$C-urea breath test, and there was no significant difference in eradication rates of *H. pylori* infection among the groups. Another group of researchers studied cMDLf in combination with quadruple antibiotic therapy in *H. pylori*-infected patients who experienced failure of a first antibiotic therapy (Tursi et al., 2007). Seventy patients were enrolled in the study and randomized into one

of two groups: 1) Group A: ranitidine bismuth citrate (400 mg 2×/d), esomeprazole (40 mg/d), amoxicillin (1 g 3×/d), tinidazole (500 mg 2×/d) without cMDLf for 7 d (n=35) or 2) Group B: the quadruple antibiotic therapy plus cMDLf (200 mg 2×/d; 400 mg/d total) for 7 d (n=35). Elimination of *H. pylori* infection was achieved in 88.57% of patients in group A and 94.28% of those in group B; the difference between groups was not statistically significant. Regarding the overall tolerability of the therapy, 16/68 patients (23.53%) exhibited side effects, but analysis of the side effects in the two groups revealed that cMDLf supplementation reduced the side-effect incidence. One group A patient withdrew from the study due to severe side effects (vomiting, diarrhea, abdominal pain) and nine other patients from this group (26.47%) experienced side effects but completed the study. Six (17.64%) group B patients experienced side effects and all completed the study.

Kondo and co-workers administered either a placebo (n=10) or 1.8 g of Morinaga Milk Industries' cMDLf (n=8)/day to patients with chronic periodontitis for three mo (Kondo et al., 2008). Only an English abstract is available; however, no adverse effects of cMDLf are reported. At one wk, *Porphyromonas intermedia* was significantly reduced in the subgingival plaque in the cMDLf group, versus control ($p<0.05$) and *P. gingivalis* was also reduced at 1 and 3 mo in the treatment group, versus control ($p<0.05$). There was also a reduction in the total number of bacteria observed in the subgingival plaque after one month in the cMDLf group, versus control ($p<0.01$).

Ishikado and co-workers studied the effect of cMDLf in patients with periodontal disease in an open intervention study (Ishikado et al., 2010). Fourteen patients aged 37-to 59-yr-old having periodontal disease were given 180 mg cMDLf/day as tablets that were formulated using liposomes from soy phosphatidylcholine for four wks (the authors report that soy phosphatidylcholine may contribute synergistically to the anti-inflammatory effects of cMDLf). cMDLf was supplied by Morinaga Milk Industry. Two subjects were dropped from the study due to lack of compliance. There were no clinically adverse effects on biochemical or hematological parameters examined, and any variations that occurred were reported to be within normal historical ranges (data for these parameters not reported). Levels of monocyte chemoattractant protein-1 (MCP-1) in gingival crevicular fluid were significantly decreased at 2 and 4 wks, versus baseline ($p<0.001$ and $P<0.001$, respectively). At four wks, the inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) were reduced ($p<0.05$), as were IL-1β and MCP-1 ($p<0.01$), in the culture supernatant of *Porphyromonas gingivalis* lipopolysaccharide (LPS)-stimulated peripheral blood monocytes from subjects, versus baseline levels.

Yamauchi and co-workers report the only published results on the effects of oral cMDLf on a topical fungal infection, *Tinea pedis* (Yamauchi et al., 2000a). Adults having mild or moderate *Tinea pedis* were assigned to one of three groups: 1) 600 mg cMDLf/day (n=14), 2) 2000 mg cMDLf/day (n=12), or 3) placebo (n=11) for 8 wks. Although a mycological cure was not observed in any of the subjects, the dermatological symptoms score was decreased for both cMDLf groups versus control ($p<0.05$). There were no adverse events reported and no subjects withdrew from the study because of an adverse event.

Mueller and co-workers administered cMDLf to subjects having mild to moderate facial acne vulgaris in an open label study (Mueller et al., 2011). Forty-three healthy subjects aged 17.5 ± 3.8-yr-old, were enrolled in the study and those with known milk intolerance or allergy were excluded. Subjects ingested 200 mg of cMDLf in the form of chewable tablets/day for eight wks. Compliance was > 95% and no serious adverse events or adverse events leading to discontinuation of the cMDLf ingestion were reported. None of the subjects experienced an adverse event considered to be related to the test material. The total count of acne lesions was significantly decreased from baseline at 2, 4, and 8 wks' time ($p=0.005$, 0.007, and <0.001, respectively). In particular, inflammatory lesions were reduced at 2 wks ($p=0.002$), while non-inflammatory lesions were reduced at 4 and 8 wks ($p<0.001$ for both), versus baseline.

Five studies were retrieved that examined the effects of oral supplementation with Morinaga's cMDLf in patients with chronic hepatitis C (Iwasa et al., 2002; Okada et al., 2002; Ishii et al., 2003; Konishi et al., 2006; Ueno et al., 2006). Hepatitis C virus-related cirrhotic patients with persistently high serum levels of alanine aminotransferase (ALT) activity have higher risk of development of hepatocellular carcinoma (HCC) than those with persistently low levels of ALT activity. Additionally, animal models have shown mitochondrial injury, hepatic steatosis and hepatic carcinogenesis with increased free radicals in the liver. cMDLf, has been shown in the literature to inhibit lipid peroxidation induced by oxidative stress and have an inhibitory effect against HCV in cell culture as well as improve serum ALT levels in patients (Knoshi et al. 2006). Therefore, studies to further explore the use of cMDLf in patients with hepatitis C have been conducted.

Iwasa and co-workers enrolled 27 patients who had elevated ALT levels, were serum-positive for anti-HCV antibody, plus high serum titers of HCV RNA and serum HCV genotype 1b and randomized subjects to receive either 1) 0.4 g cMDLf/day (n=10) or 2) 3.6 g cMDLf/day (n=15) for 6 mo (Iwasa et al., 2002). All subjects completed the study and the authors reported no serious complications occurred during treatment. At 2, 4, and 6 mo, HCV RNA levels were

significantly reduced ($p<0.05$, 0.01 and 0.01, respectively) in the high-dose cMDLf group, versus baseline levels. Two mo after the study ended, HCV RNA levels had increased but not yet returned to baseline levels.

Okada and co-investigators treated 45 patients with confirmed chronic hepatitis C (based on biopsy, genetic and biochemical criteria) with 1.8 (n=15), 3.6 (n=15), or 7.2 g (n=15) cMDLf/day for 8 wks (Okada et al., 2002). Authors reported that there were no clinically significant abnormalities in laboratory values observed during or after treatment in this study (methods state that a complete blood count, biochemistry tests, urinalysis and serum tests were performed, but give no details or data). No serious adverse events were reported. Adverse events that appeared to be dose-related occurred in four subjects and included diarrhea in one person in the 3.6 g group, plus three people in the 7.2 g group who experienced skin eruption, anorexia, fatigue, chills and constipation. No adverse effects were found in persons at the lowest dose. A virological response was observed in four patients in the 1.8 g group at the end of treatment but they still had persistent and detectable serum HCV RNA levels. The duration of response ranged from 6-10 mo. All patients who responded to cMDLf relapsed during the follow-up period but an age-associated effect was observed wherein patients ≥ 60-yr-old had higher response rates versus those younger than 60-yr-old ($p<0.01$). A biochemical response in the form of lowered ALT levels was observed in two patients, one in the 1.8 g/d group and one in the 3.6 g/d group, and the effect persisted 5-6 mo. None of the subjects achieved ALT normalization.

Ishii and co-authors reported on the effect of supplementation with cMDLf plus lactulose plus *Bifidobacterium longum* in patients with chronic hepatitis C (Ishii et al., 2003). Sixty-three patients who were serum-positive for anti-HCV antibodies and HCV RNA were randomized to receive either 1) 600 mg cMDLf plus 600 mg lactulose plus 3 billion active *B. longum*/day (n=36) or 2) no cMDLf (n=27) for 12-36 mo. It was assumed that the control group received no interventions, including the lactulose and *B. longum* supplement, although this was not explicitly stated in the published report for this study. The methods did not specifically mention recording adverse effects; however, no adverse findings were reported. At three mo, there was a significant increase in serum IL-18 levels, versus baseline, in the treatment group ($p<0.01$); this effect was transient. There was no effect on serum ALT, HCV RNA, and IL-10 levels, or on CD4+ Th1 and CD4+ Th2 cells.

Konishi and co-workers examined the effect of cMDLf on lipid peroxidation in chronic hepatitis C patients having high serum HCV RNA, HCV genotype 1b and who did not have other causes of liver dysfunction (Konishi et al., 2006). Ninety patients received either 3.6 g cMDLf/day (n=47) or no intervention (n=43) for 8 wks. By the end of the study, the cMDLf-treated group had reduced serum ALT levels ($p<0.05$) and reduced plasma levels of 8-

isoprostane ($p<0.05$) (a marker of lipid peroxidation), versus baseline. The methods did not specifically mention recording adverse effects; however, no adverse findings were reported.

Ueno and co-investigators carried out the largest study of cMDLf in chronic hepatitis C patients, enrolling 199 patients with adequate bone marrow and renal function, and assigning them to receive either 1) 1.8 g cMDLf/day (n=97) or 2) placebo (n=101) for 12 wks (Ueno et al., 2006). Three subjects withdrew for reasons that were unrelated to adverse effects but not specified. Minor adverse events occurred with similar frequency and intensity across groups and included neutropenia, γ-GTP elevation and hyperglycemia. CMDLf was well tolerated and no serious adverse events were reported during treatment. cMDLf treatment did not significantly affect the virologic or biochemical response rates; nor did it alter serum IL-18 levels or CD4+, CD8+, CD16+ or CD56+ peripheral blood lymphocyte populations, versus control.

There are also studies that have investigated the effect of cMDLf on the colonization of children by pathologic microbial species (Okuda et al., 2005; Egashira et al., 2007; Zuccotti et al., 2007; Ochoa et al., 2008). The Okuda study was summarized above.

Egashira and co-workers enrolled 298 children under 5-yr-old who were attending nursery school or kindergarten and assigned them to one of two groups: 1) 100 mg lactoferrin/day for 12 wks (n=136) or 2) no cMDLf-containing products (n=98) for 12 wks (Egashira et al., 2007). Milk allergic children were excluded. cMDLf was supplied by Morinaga Milk Industry. Although the number of children with rotaviral gastroenteritis was similar between treatment groups, the frequency and duration of vomiting (p=0.0106, 0.0137) and diarrhea (p=0.0446, 0.0285) were significantly reduced in the cMDLf group, versus control. Two children in the control group were hospitalized due to dehydration, compared with none in the cMDLf group.

The Zuccotti study examined the immune modulating effects of cMDLf in eleven human immunodeficiency virus (HIV)-infected, antiretroviral-therapy-naïve children aged 4-17 yr (Zuccotti et al., 2007). Subjects received 1 g cMDLf every 8 hours daily for 4 wks (3 g/day total). The methods did not specify recording of adverse effects, however, there was no report of treatment-related adverse effects due to cMDLf. However, cMDLf supplementation did reduce (no statistics) the percent naïve and central memory CD4+ and CD8+ T cells and increase populations of terminally differentiated lymphocytes at 4 wk, versus baseline. cMDLf supplementation also significantly improved the ability of CD13+ cells to ingest and kill (p=0.01 and 0.009, respectively) labeled *Candida albicans* blastospores at 4 wk, versus baseline. CMDLf

also significantly increased the IL-12:IL-10 ratio in lipopolysaccharide (LPS)-stimulated CD14+ cells (p=0.001) at 4 wk, versus baseline. These immune modulations may be beneficial in HIV positive subjects.

Ochoa and co-workers enrolled previously weaned children 12- to 36-mo-old in a study and randomized the subjects to receive either 0.5 g cMDLf twice/day for six d/wk (daily average: 0.86 g/d) (n=146) or 0.5 g maltodextrin twice/day for six d/wk (n=174) for nine mo (Ochoa et al., 2008). Children with personal or family history of cow's milk or infant formula allergy, milk intolerance or moderate-to-severe allergic rhinitis or asthma were excluded from the study. There were no adverse events related to cMDLf ingestion reported in this study and height-for-age scores were significantly higher in the cMDLf group when analyzed by group (p=0.03) and by interaction of group and month (p=0.03). There was no difference in weight for age scores. At nine mo, there was a significant reduction in the number of positive stool samples for *Giardia lamblia* in the cMDLf group, versus the control group (p<0.05). This was the only study to report an effect of oral ingestion of cMDLf on colonization by a protozoal parasite.

## 4. CLINICAL STUDIES OF cMDLf IN ADULT WOMEN INVESTIGATING EFFECT ON IRON HOMEOSTASIS

Published studies suggest that oral ingestion of cMDLf may be beneficial for maintaining iron homeostasis. Clinical studies examining this endpoint also provide some information relevant to the corroboration of safety of intake of cMDLf.

Koikawa and co-workers carried out a double-blind, randomized trial that examined the ability of cMDLf to improve or prevent anemia in female long distance runners at high risk of iron-deficiency anemia (Koikawa et al., 2008). Sixteen women (approximately 20-yr-old) were randomized to either 1.8 g cMDLf (as tablets containing 0.45 g cMDLf each) plus 6 mg iron (as ferric pyrophosphate)/day (n=8) or iron supplement only (n=8) for eight wks. All subjects also received 30 g of hydrolyzed milk protein in 700 mL water daily. The cMDLf was supplied by Morinaga Milk Industry and was 17% iron-saturated, with 0.23 mg iron/gram of protein. At the end of the study, cMDLf-supplemented runners did not have the significant decrease in serum iron and ferritin levels, as was observed in the control group. Compared with the control group, cMDLf-supplemented runners had significantly increased red blood cell (RBC) count (p<0.01) by 8 wk. At 8 wk, the cMDLf-supplemented runners had significantly elevated mean corpuscular volume (MCV) (p<0.01) and mean corpuscular hemoglobin (MCH) (p<0.05) values, versus baseline levels, although final values did not differ significantly from those observed in the control group.

Nappi and co-workers studied the effect of cMDLf on iron status and tolerability in pregnant women with iron deficiency anemia (Nappi et al., 2009). The investigators evaluated 139 pregnant women having a physiological course of a singleton pregnancy and who were between 12 and 36 wks gestation with iron deficiency anemia as defined by hemoglobin (Hb) value < 11 mg/dL, serum iron < 30 μg/dL, serum ferritin < 12 μg/dL and total iron binding capacity (TIBC) > 450 μg/dL. One hundred women were enrolled and randomized to receive either 100 mg cMDLf/day (n=50) or 520 mg ferrous sulfate (n=50) for 30 d. Both cMDLf and ferrous sulfate supplementation caused a significant increase in Hb, serum ferritin, serum iron and a concurrent decrease in TIBC at 30 d, versus baseline levels ($p<0.01$). Median scores of abdominal pain and constipation were significantly higher in the ferrous sulfate group compared with the cMDLf group, and cMDLf was reported to be well tolerated. In the cMDLf group, gastrointestinal side effects including epigastric pain, vomiting and constipation were reported for one subject for each side effect. One patient in the cMDLf group was excluded due to miscarriage and two subjects from the ferrous sulfate group discontinued treatment due to severe constipation.

Paesano and co-workers have studied the effects of cMDLf on iron parameters in pregnant and non-pregnant women (Paesano et al., 2006; Paesano et al., 2009; Paesano et al., 2010). One study included 300 pregnant women of various ages, parities and trimesters of pregnancy who were offered oral iron supplementation (Paesano et al., 2006). Pregnant women who refused treatment represented the control group (n=54), and the remaining subjects were randomly divided into two groups, receiving 520 mg ferrous sulfate/day (n=98) or 100 mg cMDLf twice/day (200 mg total/d) (n=107). The cMDLf was 30% iron-saturated. The interventions were administered for 30 d and hemoglobin and total serum iron levels were measured at 0 and 30 d. It should be noted that, although the study authors define iron deficiency as Hb ≤ 11 g/dL and iron deficiency anemia as total serum iron of ≤ 30 mg/dL, most of the women enrolled in this study met neither of these criteria. However, oral ingestion of 200 mg cMDLf/day caused a significant increase in the delta mean values for both Hb and total serum iron in this group, versus control ($p<0.01$ for both) and versus ferrous sulfate ($p=0.02$ for Hb effect; $P<0.01$ for total serum iron effect) groups. The authors report that no side effects were observed in the 107 women who received cMDLf. In 98 women receiving ferrous sulfate, the authors report that 95% had stomach pain, cramps and constipation; 2% had at least one episode of diarrhea. Thirty-one subjects were excluded or lost to analysis (reason not specified), 4 moved or were lost to follow-up, 3 had miscarriages, and 3 were excluded for other reasons not stated.

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A second study by the same investigators was reported as part of a review paper, and thus many details are not available, such as the inclusion and exclusion criteria, study protocol, randomization scheme, and any adverse effects (Paesano et al., 2009). There was no reference

made in the review article to a published report that could be retrieved. One hundred forty-three pregnant women either refused therapy (n=33), or were given cMDLf (n=60; dose not stated, presume 200 mg/d, as in previous study) or ferrous sulfate (n=50) for 30 d. Mean values for red blood cell count (RBC), Hb, total serum iron and serum ferritin were increased in the cMDLf group at 30 day, versus baseline levels ($p<0.001$ for all).

A third report from Paesano and co-workers was a publication of results from two studies, one in pregnant women and the second in non-pregnant women (Paesano et al., 2010). The first study involved pregnant women in their third trimester of pregnancy who met at least one of the following hematological criteria: $< 4\times 10^6$ RBC/mL, Hb $\leq$ 11 g/dL, total serum iron $\leq$ 30 mg/dL or serum ferritin $\leq$ 12 ng/mL. Twelve pregnant women refusing iron therapy were designated as the control group and the remaining 75 women were assigned to receive either 520 mg ferrous sulfate once/day (n=33) or 100 mg cMDLf twice/day (200 mg total/d) (n=30) for 30 d. After 30 d, mean RBC, Hb, total serum iron, serum ferritin and hematocrit levels increased significantly in the cMDLf group, versus baseline levels ($p<0.001$ for all parameters). The authors reported that there were no dropouts in the cMDLf group. The second study enrolled non-pregnant women meeting the same hematological criteria as the first study and excluded subjects who were allergic to milk proteins. Women were offered iron therapy and the nine women refusing intervention constituted the control group. The remaining 189 women consenting to iron therapy were randomized to receive 520 mg ferrous sulfate once/day (n=90) or 100 mg cMDLf twice/day (200 mg total/day) (n=90). Of the subjects receiving ferrous sulfate, therapy was administered for 30 (n=25), 60 (n=25), or 90 d (n=26). Of the non-pregnant women receiving cMDLf, the intervention was carried out for either 30 (n=34), 60 (n=22), or 90 d (n=34). After 30, 60 and 90 d, mean RBC, Hb, total serum iron, serum ferritin and hematocrit levels increased significantly in the cMDLf group, versus baseline levels ($p<0.001$ for all parameters). The study authors report that there were no drop-outs in the cMDLf group due to side effects.

Results from the above studies suggest that cMDLf may exert favorable effects on iron homeostasis in women, although in some of the published studies non-standardized hematological inclusion criteria were utilized. Administration of up to 1.8 g cMDLf/day for 8 wks in female long distance runners and 200 mg cMDLf/day for up to 30 d in pregnant women was well tolerated.

## 5. INFANTS

### a) Clinical studies in term and preterm infants

Several studies have been published on the effects of oral ingestion of cMDLf in infants, at formula concentrations ranging from 10 mg/100 mL (0.01%) to 285 mg/100 mL (0.285%), at durations ranging from two wks to one yr. Five studies were retrieved that enrolled healthy term infants (Fairweather-Tait et al., 1987; Chierici et al., 1992; Roberts et al., 1992; Hernell and Lönnerdal, 2002; King et al., 2007) and three studies were carried out in low birth weight infants (Kawaguchi et al., 1986; Kawaguchi et al., 1989; Manzoni et al., 2009). Results from the studies suggest that supplementation of infant formula with cMDLf is safe and well tolerated by infants at levels up to 0.285% (w/v).

The highest exposure to cMDLf was found in the Fairweather-Tait and co-workers' study, where 36 healthy, full-term (36-41 wk) infants weighing 2060-3800 g received either milk-based formula with (285 mg/100 mL; 86 μg total iron) or without (40 μg total iron) cMDLf for 14 d (Fairweather-Tait et al., 1987). This was a stable isotope feeding study that was part of a larger investigation into the effects of cMDLf on fecal flora. After 7 d on control formula, these infants were orally administered either 40 or 86 μg $^{58}$Fe as $^{58}$Fe-ferric chloride plus ascorbic acid (n=8 and *n*=8, respectively) and all subsequent diapers were collected for the following 3 d. Infants who received cMDLf-supplemented formula for 7 d were administered either 40 or 86 μg $^{58}$Fe as $^{58}$Fe-cMDLf (n=8 and *n*=5, respectively). Reported study exclusions included noncompliance (one subject), administration of incorrect feed (one subject), inadvertent pooling of fecal material (three subjects), and chronic constipation (two subjects). The methods did not specify recording of adverse effects, however, there was no report of treatment-related adverse effects.

Lonnerdal and Hernell studied the effects of iron, copper, and selenium supplementation of infant formula on growth, and iron, zinc, and copper status in infant from 6-wk- to 6-mo-old (Lonnerdal and Hernell, 1994). The target iron level for each formula was 4 mg/L delivered either as $FeSO_4$ or bovine lactoferrin in combination with $FeSO_4$ (1.2 mg/L bovine lactoferrin and 2.6 mg/L $FeSO_4$). There were no differences in weight or height at 6 wk or 6 mo between the breast-fed and formula-fed groups. However, serum selenium, α2-macroglobulin, and glutathione peroxidase levels were reduced in the supplemented formula fed groups compared to those fed breast milk, but there were no differences in any of these parameters among the different formulas. All other hematological indices were similar between infants consuming breast milk or infant formula supplemented with bovine lactoferrin. All infants had a satisfactory iron status at the end of the treatment period.

Hernell and Lonnerdal studied the effects of cMDLf administration on iron parameters in healthy term infants that were initially breast-fed and then allocated to either a breast-fed group (n=16) or various formula-fed groups (n=10-12), according to parent choice (Hernell and Lönnerdal, 2002). Experimental formulas contained 1.6, 1.8, 2.2, or 4.0 mg iron/L, where iron was present as $FeSO_4$, except in the 1.8 mg iron group, where 1.3 mg Fe was contributed from added cMDLf and 0.5 mg was present as $FeSO_4$. The 2.2 mg Fe group also received 40 mg/L monophosphate nucleotides. The cMDLf ingredient was reported to have a protein content of 95% (w/w), of which cMDLf constituted > 95%. The cMDLf was iron-saturated and had an iron content of 1.24 mg/g protein. Therefore, the concentration of cMDLf present in the supplemented formula was calculated as 0.104 g/100 mL, or 0.104% (w/v). Experimental formulas were fed to the infants for six mo, at which time there was no observed effect of cMDLf on body weight or height, except for a significantly greater weight in infants in the $Fe^{2+}$ with cMDLf group compared to the group fed 2.2 mg Fe plus nucleotides ($p<0.05$) at 6 mo of age. All formulas were reported to be well tolerated.

Chierici and co-workers studied the effect of cMDLf supplementation on serum hemoglobin, hematocrit, ferritin, iron and zinc levels in healthy, full-term infants (Chierici et al., 1992). Fifty-one infants were assigned (protocol not provided) to one of four groups: breast milk (n=10), control formula (n=13), formula containing 10 mg cMDLf/100 mL (0.01%, w/v; *n*=14), and formula containing 100 mg cMDLf/100 mL (0.1%, w/v; *n*=14). The cMDLf was non-heat-treated and 20% iron-saturated. The iron content of the 100 mg cMDLf formula was slightly higher (98 µg/100 mL) versus the control formula (70 µg/100 mL) and the 10 mg cMDLf formula (72.8 µg/100 mL). Breast milk or formulas were fed for 150 d. The methods did not specify recording of adverse effects, however, there was no report of treatment-related adverse effects.

Roberts and co-workers administered an experimental formula containing either 10 (0.01%, w/v; *n*=15) or 100 mg (0.1%, w/v; *n*=14) cMDLf/100 mL to healthy full-term infants (Roberts et al., 1992). Unsupplemented formula (n=14) and breast milk (n=12) were fed in the two control groups. The cMDLf was 20% iron-saturated. Formulas were fed for 3 mo. The purpose of the study was to monitor the effects of cMDLf supplementation on infant fecal flora. The methods did not specify recording of adverse effects, however, there was no report of treatment-related adverse effects.

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King, Jr. and co-workers examined the impact of cMDLf supplementation in healthy infants 0- to 4-wks-old (≥ 34 wks' gestation; ≥ 2000 g birth weight) who were strictly bottle-fed (King et al., 2007). Infants were randomized to receive powdered Similac with iron formula (3 mg/L elemental iron) with (850 mg/L; 0.085%, w/v; *n*=26) or without (102 mg/L; background

cMDLf content of cow's milk, 0.0102%, w/v; *n*=26) added cMDLf for twelve mo. The cMDLf contained 120 μg of iron/gram of powder. The authors noted that, as protein content was not normalized across the two infant formulas, the cMDLf group received ~5% additional protein versus the control group, which may or may not have influenced the growth of these infants. There was a trend for increased weight over time, up to 6 mo, in the cMDLf-supplemented group, but this increase did not reach statistical significance ($p=0.06$). Infants given cMDLf-supplemented formula had a significantly increased hematocrit at 9 mo ($p<0.05$) and a trend toward increased mean corpuscular volume (MCV) at 12 mo ($p=0.06$), versus the control group. Twenty-seven infants dropped out of the study; 13 of these received the cMDLf-supplemented formula. Of the 27 drop-outs, nineteen withdrew due to intolerance; of these 19 intolerant infants, 10 received cMDLf-supplemented formula. Three infants were lost due to withdrawal of consent and five were lost to follow-up. These results suggest that, when supplemented at 0.085% (w/v) in infant formula, cMDLf does not increase the risk for intolerance in healthy infants.

Kawaguchi and co-workers conducted two studies on the effects of cMDLf in low birth weight infants (Kawaguchi et al., 1986; Kawaguchi et al., 1989). In the earlier study, 16 low birth weight infants weighing > 1500 g were fed with commercially available standard infant formula products until stable feeding of 150 mL/kg/day was achieved (Kawaguchi et al., 1986). Then the standard formula was replaced with formula containing Morinaga Milk Industries' cMDLf at 50 mg/100 g powder (assuming 13 g powder + 93 g water to reconstitute, the calculated final concentration is 6.5 mg cMDLf/100 mL formula) for 2 wks, after which the formula was switched back to a commercially available infant formula. Assuming the intake pattern of formula was stable over the course of the study, an intake of cMDLf of 9.75 mg/kg body weight/day can be calculated. The study authors report that, during the cMDLf feeding period, feces tended to become soft. Additionally, cMDLf was detected in the feces at a concentration of 20-750 μg/gram of feces. There was no mention of adverse events. In the later study, nine premature, low birth weight infants (1454-2034 g; 29-36 wk gestational age) who were being bottle fed were supplemented with Morinaga's cMDLf in infant formula at 100 mg/100 mL (0.1%, w/v) for 2 wks (Kawaguchi et al., 1989). Stable bottle-feeding was reported to be achieved again at approximately 150 mL/kg body weight/day, or 150 mg cMDLf/kg body weight/day. There was a one-wk follow-up period after the experimental formula was discontinued. No adverse events were mentioned. In this second study, 8-9 mg of cMDLf was detected/gram of feces, which is higher than reported in the previous study due to the larger dose of cMDLf administered.

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Manzoni and co-workers studied the ability of cMDLf to affect the incidence of late-onset sepsis in very low birth weight neonates (Manzoni et al., 2009). Very low birth weight

neonates (<1500 g) younger than 3-d-old were enrolled and randomized to receive placebo (2 mL of a 5% glucose solution; n=168), 100 mg cMDLf/day (n=153) or 100 mg cMDLf plus $6 \times 10^9$ CFU *Lactobacillus rhamnosus* GG/day (n=151) for either four (birth weight 1001-1500 g) or six (birth weight < 1000 g) wks. Effectively, infants were treated from birth until day 30 or 45 of life. Treatments were diluted in prepared milk prior to administration. Breastfeeding was encouraged and supplemented when necessary with a very low birth weight formula that did not contain cMDLf. Adverse events were recorded until 2 d after the end of the study and liver function was evaluated wkly. Mortality attributable to sepsis was significantly reduced in the cMDLf-supplemented group, relative to control ($p$=0.008). No adverse effects or intolerances to treatment occurred. One infant in the cMDLf group discontinued treatment after eight doses (reason not provided), but was included in the intent-to-treat analysis. Nine infants had incomplete data on some variables but not for analysis of study outcomes. The incidence of hyperbilirubinemia requiring phototherapy was similar across the three groups and no infants exhibited signs of hepatotoxicity or cholestasis. Due to the range of birth weights of the infants that were enrolled in this study, the/body weight exposure to cMDLf can be estimated at 66.7 to greater than 100 mg/kg body weight/day.

**b) Effects on maintenance of a healthy microflora**

Five published studies of cMDLf in infants reported results of intake on endpoints relating to establishment of a healthy gut/fecal flora or prevention of infection (Kawaguchi et al., 1986; Kawaguchi et al., 1989; Roberts et al., 1992; King et al., 2007; Manzoni et al., 2009); two of these administered Morinaga Milk Industries' cMDLf (Kawaguchi et al., 1986; Kawaguchi et al., 1989).These studies indicate that no adverse impact on microflora has been demonstrated and in fact, a possible beneficial effect of cMDLf consumption on gut flora is seen.

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Kawaguchi and co-workers carried out two studies in low birth weight infants. The first study by Kawaguchi and colleagues (1986), previously discussed, reported that during the cMDLf supplementation period, fecal pH declined (second wk); fecal lysozyme activity increased; organic acid content of feces increased (especially acetic acid during the second wk); and the ratio of Bifidobacteria among total bacteria tended to be higher, while the Staphylococcus detection ratio tended to be lower (no statistics provided). The second study by the same investigators (1989), also previously discussed, noted that by the end of the 2 wk feeding period, the composition ratio of *Bifidobacterium* and *Veillonella* culturable species in the fecal flora had increased relative to baseline levels (no statistics). Conversely, the composition ratio of *Enterobacteriaceae* and *Clostridium* declined during the same period. Both effects persisted and were observable by 1 wk after discontinuation of the cMDLf supplementation. *Staphylococcus* and *Clostridium* species were not detected at 1 wk post-completion of the study.

The relative ratio of *Bacteroidaceae* species was increased at the end of the study but this was a transient effect that disappeared by the end of the one-wk period after discontinuing cMDLf. No other effects were noted on fecal pH or organic acid content (acetic, lactic acids).

Roberts and co-investigators studied the effect of cMDLf on the fecal flora of healthy, full-term infants (Roberts et al., 1992). As previously discussed, healthy term infants were administered a 20% iron-saturated cMDLf preparation, supplemented in formula at 10 and 100 mg/100 mL until the third month of life. At the end of the study, 57% of infants in the high-dose cMDLf group exhibited a "bifidus fecal flora," defined as a flora where bifidobacteria outnumber all other components of the fecal flora by 1 $\log_{10}$ CFU/g of feces, versus 0% at baseline (no statistics). This level was similar to the prevalence of bifidus flora observed in the breast-fed group (50%); the effect was not observed in the low-dose cMDLf group. These two studies suggest that cMDLf may exert a beneficial effect on the ecological balance of infant gut flora.

Two studies reported the effects of orally supplemented cMDLf on the incidence of various infections in neonates (King et al., 2007; Manzoni et al., 2009). As previously discussed, King and co-workers enrolled healthy infants 0- to 4-wks-old given formula with or without added cMDLf for 12 mo (King et al., 2007). At the end of the study, cMDLf-supplemented infants had significantly lower occurrence of lower respiratory infections versus the control group ($p<0.05$). The Manzoni study enrolled very low birth weight neonates younger than 3-d-old and assigned subjects to one of three treatment groups including a cMDLf supplemented formula group, a placebo and a cMDLf group with added probiotic, for either 4 or 6 wks. cMDLf alone significantly reduced mortality attributable to sepsis ($p=0.008$), total late-onset sepsis ($p=0.002$), sepsis due to Gram-positive bacteria ($p=0.007$), sepsis due to Gram-positive bacteria, including episodes diagnosed with $\geq 1$ positive blood culture for coagulase-negative *Staphylococcus* species ($p=0.002$), sepsis in extremely low birth weight neonates ($p=0.002$), sepsis in neonates weighing $\leq 750$ g ($p=0.003$), sepsis in neonates $\leq 27$ wks gestational age at birth ($p=0.01$), total incidence of invasive fungal infection ($p=0.004$), invasive fungal infection in extremely low birth weight neonates ($p=0.03$), and progression rate from colonization to invasive fungal infection in all neonates ($p=0.002$), versus control. Outcomes for bacteria-related endpoints were similar in the group treated with cMDLf plus probiotic; however, this treatment was not as effective against fungal colonization and infection, for unknown reasons. Treatment with cMDLf was most effective in the low birth weight infants weighing $\leq 750$ g, which corresponds to a dose of $\geq 133$ mg/kg/day. Results from these two studies suggest that oral supplementation with cMDLf may be beneficial in infants in the context of certain microbial infections.

**c) Effects on iron homeostasis**

Three published studies reported either no effect or a beneficial effect of orally administered cMDLf on maintenance of iron homeostasis in infants. In the Fairweather-Tait and co-workers' study (previously discussed), 36 healthy, full-term infants were given formula supplemented with cMDLf for 14 d (Fairweather-Tait et al., 1987). This was a stable isotope feeding study that was part of a larger investigation into the effects of cMDLf on fecal flora. After 7 d on control formula, these infants were orally administered either 40 or 86 μg $^{58}$Fe as $^{58}$Fe-ferric chloride plus ascorbic acid (n=8 and *n*=8, respectively) and all subsequent diapers were collected for the following 3 d. Infants who received cMDLf-supplemented formula for 7 d were administered either 40 or 86 μg $^{58}$Fe as $^{58}$Fe- cMDLf (n=8 and *n*=5, respectively). There were no differences across groups regarding fecal iron concentration or total iron excreted following administration of labeled iron substrates.

Chierici and co-workers studied the effect of cMDLf supplementation on serum hemoglobin, hematocrit, ferritin, iron and zinc levels in healthy, full-term infants (previously discussed) (Chierici et al., 1992). Infants were assigned to one of four groups: breast milk, control formula, formula containing 10 mg cMDLf/100 mL (0.01%, w/v), and formula containing 100 mg cMDLf/100 mL (0.1%, w/v). Breast milk or formulas were fed for 150 d and serum samples were taken at 0, 7, 30, 90, and 150 d. Two breast-fed infants had low hemoglobin levels at 90 d. There were no statistically significant differences in hemoglobin or hematocrit between the groups at any sampling time (data not shown). At 150 d, the high-dose cMDLf group had significantly higher serum ferritin versus the control formula group (p=0.02), in spite of an across the board drop in serum ferritin in all groups. However, the high-dose cMDLf group received slightly more iron versus the other two formula groups, which may or may not account for the difference. Breast-fed infants had lower serum iron levels compared with infants fed the control formula (p=0.012) and the high-dose cMDLf group (p=0.041). There also were no significant differences in serum zinc levels across all groups at any sampling time.

Hernell and Lonnerdal studied the effects of cMDLf on iron parameters in healthy term infants that were initially breast-fed and then allocated to either a breast-fed group or various formula-fed groups, including one with iron plus cMDLf, according to parent choice (previously discussed) (Hernell and Lönnerdal, 2002). Experimental formulas were fed to the infants for six mo, at which time there was no effect on iron parameters, including serum iron, ferritin, hemoglobin, mean corpuscular volume, total-iron-binding capacity, or transferrin receptor, at 1, 4 or 6 mo among all groups with the exception of significantly lower serum iron for the iron plus nucleotide supplemented formula group compared to other formula groups at 1 month only. No significant differences in serum zinc and copper concentrations were observed among groups at 1, 4 or 6 month.

| Table 19. Human Clinical Studies of cMDLf– Adults | | | | |
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| **Reference** | **Objective/Test Article** | **Study Design** | **Outcome** | **Safety-Related Observations Reported** |
| (Yamauchi et al., 1998) | To study the effects of orally administered cMDLf on the immune system of healthy volunteers.<br><br>*Study type:* Intervention-only<br><br>*Source:* cMDLf was from Morinaga Milk Industry Co., Ltd., Japan | Ten healthy male volunteers, aged 31-55 yr, were given 2 g cMDLf/day for 4 wk. Blood samples were drawn before, during and after the intervention. Phagocytic activity and superoxide production activity of polymorphonuclear leukocytes (PMN) were evaluated and the expression levels of CD11b, CD16 and CD56 on leukocytes were quantified on leukocytes using flow cytometry. | • Phagocytic activity of PMN increased (no statistics) in 3/10 subjects during the cMDLf intervention.<br>• In two of three subjects in which the phagocytic activity increased, PMN expressed CD16 at higher levels as well.<br>• Superoxide production of PMN increased in only 1 of the 10 subjects.<br>• CD16+ lymphocytes increased in 3/10 subjects.<br>• CD11b+ lymphocytes and CD56+ lymphocytes increased in four subjects, including the same three subjects who showed an increase in CD16+.<br>• Overall, 7/10 subjects had changes in immune parameters during the cMDLf treatment. | • None |
| (Yamauchi et al., 2000a) | To evaluate the effectiveness of cMDLf in the treatment of *Tinea pedis*.<br><br>*Study type*: Double-blind, placebo-controlled, randomized<br><br>*Source*: Milei GmbH, Leutkirch Adrazhofen, Germany | Adult subjects (age not stated) having mild or moderate *Tinea pedis* confirmed by microscopy and culture were assigned to one of three groups: 1) 600 mg cMDLf (cMDLf)/d (n=14), 2) 2000 mg cMDLf/d (n=12), and 3) placebo (n=11). Four tablets of either low- (75 mg/tablet) or high-dose cMDLf (250 mg/tablet) were administered twice a day for 8 wk. Each subject's feet were inspected 1 wk prior to initiating treatment, at 2, 4, and 8 wk after beginning treatment, and 4 wk after ending treatment. Clinical symptoms were evaluated and recorded, and cultures were taken. Immune parameters, hematology tests, blood chemistry and urinalysis were also measured. | • The dermatological symptoms score was statistically significantly decreased for both cMDLf treatment groups ($p<0.05$), versus control, at 12 wk.<br>• The above improvements were noted in a subgroup of patients that excluded those having an initial symptom score of under five, or those having "hyperkeratosis type" *Tinea pedis*. Eight subjects comprised the control group, eight subjects were included from the 600 mg cMDLf/d group and five subjects were included from the 2000 mg cMDLf/d group. | • There were no adverse events and no subjects withdrew from the study due to adverse events. |

0001104

| Table 19. Human Clinical Studies of cMDLf– Adults | | | | |
|---|---|---|---|---|
| **Reference** | **Objective/Test Article** | **Study Design** | **Outcome** | **Safety-Related Observations Reported** |
| | | | • Mycological cure was not observed in any of the subjects.<br>• At 8 wk treatment, levels of serum IgG specific for cMDLf were significantly higher than at pretreatment (data not shown), but serum levels of IgE specific for cMDLf were below the detection limit. Serum levels of cMDLf were also below the detection limit (10 ng/mL) that was also lower than the minimum inhibitory concentrations of cMDLf against *Trichophyton rubrum* and *T. mentagrophytes* (> 6 µg/mL; unpublished data). | |
| (Iwasa et al., 2002) | To assess the effect of cMDLf on hepatitis C virus viremia in chronic hepatitis C patients.<br><br>*Study design:* Double-blind, randomized<br><br>*Source:* cMDLf was from Morinaga Milk Industry Co., Ltd., Japan | Twenty-seven patients with chronic hepatitis C (16M/11F) were enrolled. Patients all had elevated ALT levels above the upper normal limit for ≥ 6 mo, were serum-positive for anti-HCV antibody, had high serum titers of HCV RNA (> 100 kIU/mL), and serum HCV genotype 1 b, plus absence of other causes of chronic hepatitis. None had responded to interferon therapy. 25 patients were randomized to receive either 1) 0.4 g cMDLf/d (n=10) or 2) 3.6 g cMDLf/d (n=15). cMDLf was administered for 6 mo. Patients were followed up for 2 mo after the study. | • HCV RNA was significantly reduced at 2 mo ($p<0.05$) and 4 and 6 mo (both $p<0.01$) in the high cMDLf dose group, versus baseline levels at 0 mo. By 2 mo post cessation of therapy, HCV RNA levels had increased but not yet returned to baseline levels. | • No serious complications occurred during the treatments and all subjects completed the study.<br>• Two patients in the low cMDLf dose group were excluded from the study due to detection of hepatocellular carcinoma soon after entry.<br>• Neither cMDLf treatment had an effect on AST or ALT levels at any time point during the study.<br>• Serum levels of aminotransesterase (? Spelling error in paper), iron and ferritin did not change at any time point in either cMDLf group (data not shown). |

**Table 19. Human Clinical Studies of cMDLf– Adults**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| (Okada et al., 2002) | To assess the effect of cMDLf on serum alanine aminotransaminase (ALT) and hepatitis C virus (HCV) RNA levels in patients with chronic hepatitis C.<br><br>*Study type*: Dose-finding, uncontrolled, treatment-only cohort study<br><br>*Source*: Morinaga Milk Industry Co., Tokyo, Japan | Patients 20- to 74-yr-old who had confirmed chronic hepatitis C and who met biopsy, genetic and biochemical criteria, were enrolled. cMDLf tablets (450 mg/tablet) were administered orally 2-3×/d for 8 wk on an outpatient basis and were followed up for an additional 8 wk. cMDLf intake and adverse events were self-recorded in a diary daily. Forty-five patients were enrolled at three dose levels (n=15/group, ×3 dose groups: 1.8, 3.6, and 7.2 g cMDLf/d). | • Biochemical response was observed in two patients (cMDLf of 1.8 g/d and 3.6 g/d) but no patient achieved ALT normalization. Duration of responses was 5 and 6 mo.<br>• Virological response was observed in four patients (cMDLf of 1.8 g/d) at the end of treatment, but had persistent, detectable serum HCV RNA levels. Duration of responses ranged from 6-10 mo.<br>• Age was associated with the response to cMDLf treatment: patients 60-yr or older had higher response rates ($p<0.01$).<br>• All patients who responded to cMDLf relapsed during the follow-up period. | • Adverse events occurred in four patients and included diarrhea (n=1; 3.6 g cMDLf/d); skin eruption (n=1; 7.2 g/d); anorexia and fatigue (n=1; 7.2 g/d); chills and constipation (n=1; 7.2 g/d). There were no clinically significant abnormalities in laboratory values during or after treatment. |
| (Di Mario et al., 2003)<br><br>0000106 | To test the efficacy of triple antibiotic therapy plus cMDLf in the eradication of *Helicobacter pylori* infection.<br><br>*Study type:* Open label, multi-arm, randomized, single-center<br><br>*Source:* cMDLf was from Dicofarm-Rome, Italy; tablets contained 100 mg/each. | One hundred fifty *Helicobacter pylori*-positive patients (76 M/74F) were enrolled in the study. Patients had dyspeptic symptoms, gastritis and peptic ulcer disease. Infection was assessed at baseline by either histology plus a $^{13}$C-urea breath test or histology plus *H. pylori* stool antigen test. Patients were randomized to receive one of the following treatments: 1) rabeprazole (20 mg 2×/d), clarithromycin (500 mg 2×/d), tinidazole (500 mg 2×/d), and cMDLf (200 mg 2×/d; 400 mg/d total) for 7d(n=51; 25M/26F; 50.1 ± 11.1 yr); 2) the antibiotic regimen alone for 7d(n=52; 26M/26F; 50.5 ± 15.3 yr); or 3) the antibiotic regimen alone for 10d(n=47; 23M/24F; 52.3 ± 15.8 yr). Subjects were considered noncompliant if < 90% of study medication was taken. | • All but 27 patients had a negative $^{13}$C-urea breath test or *Helicobacter pylori* stool antigen test by 2 mo after the end of the therapy.<br>• Eradication rates in the groups were as follows, based on intent-to-treat analysis:<br>Group 1: 92.2% (p=0.01 versus groups 2 and 3)<br>Group 2: 71.2%<br>Group 3: 70.2%<br>• Eradication rates in the groups were as follows, based on per-protocol analysis:<br>Group 1: 95.9% (p=0.005 versus groups 2 and 3)<br>Group 2: 72.5%<br>Group 3: 75.0% | • Major side effects led to treatment discontinuation in:<br>Group 1 (2 patients; dizziness, headache, fatigue, nausea)<br>Group 2 (1 patient; nausea, hypotension, taste disturbance)<br>Group 3 (3 patients; fatigue, nausea, diarrhea)<br>• All but 6 patients completed treatment with good compliance. |

**Table 19. Human Clinical Studies of cMDLf– Adults**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| (Ishii et al., 2003) | To study the effects of long-term oral administration of cMDLf on serum parameters in patients with chronic hepatitis C.<br><br>*Study design:* Randomized, controlled<br><br>*Source:* "CMDLf active R" was from Morinaga Milk Industry Co., Ltd, Japan and contained 600 mg cMDLf plus 600 mg lactulose and 3 billion active *Bifidobacterium longum* | Sixty-three patients with chronic hepatitis C (38M/25F) aged 24-77 yr were enrolled. All patients were serum-positive for anti-HCV antibodies and HCV-RNA., with an HCV genotype of 1b. Concurrent hepatitis B infection was not present. Patients were randomly assigned to one of two groups: 1) 600 mg cMDLf + 600 mg lactulose + 3 billion active *Bifidobacterium longum*/day for 12-36 mo (n=36) or 2) no cMDLf (unclear whether a placebo was given) (n=27) for 6 mo. Serum levels of alanine aminotransferase (ALT), HCV-RNA, IL-18, IL-10, CD4+ Th1 cells, and CD4+ Th2 cells were followed over time. | • There was a significant ($p<0.01$) increase in serum IL-18 at 3 mo, versus 0 mo, in the treatment group, but this effect was transient.<br>• Treatment had no effect on serum ALT, HCV-RNA, IL-10, CD4+ Th1 cells or CD4+ Th2 cells, at any measured time point during the 12 mo treatment time. | • None |
| (Okuda et al., 2005) | To study the effect of oral supplementation of cMDLf on *Helicobacter pylori* colonization in humans.<br><br>*Study type:* Randomized, double-blind, placebo-controlled<br><br>*Source:* cMDLf tablets were from Morinaga Milk Industry, Tokyo, Japan | Twenty-five healthy children and 34 healthy adults having *H. pylori* infection either without upper gastrointestinal symptoms or with minimal upper gastroinstinal symptoms who were not being treated were enrolled. *H. pylori* infection was diagnosed when both the $^{13}C$-urea breath test (UBT) and serum- or urine-based enzyme-linked immunosorbent assay (ELISA) were positive. [*Note:* It was not stated what a cutoff for a "positive" value in the UBT test might be.] Subjects who were milk intolerant were excluded. The four treatment groups were: 1) adults consuming two 100 mg cMDLf tablets twice/day (400 mg cMDLf/d) (n=17), 2) adults consuming placebo tablets (n=17), 3) children consuming two 100 mg cMDLf tablets twice/day (400 mg cMDLf/d) (n=14), and 4) children consuming placebo tablets (n=11) for 12 wk. | • The mean UBT values were significantly different at wk 0 between the two child groups ($p<0.01$), which may have introduced a greater tendency for a change in UBT to be observed, versus the adult groups.<br>• After 12 wk supplementation, 10 of 31 (32.3%) subjects in the combined cMDLf groups had a >50% decrease of their UBT value, versus baseline. One of 28 (4%) combined control subjects had a >50% decrease in their UBT value, versus baseline. [*Note:* Baseline UBT values appeared to vary widely across all groups, and it was unclear what constituted a positive result.] | • None |

000107

**Table 19. Human Clinical Studies of cMDLf– Adults**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| | | | • In most of the responders in the cMDLf groups, the UBT values returned to baseline levels by 4 wk after the end of the study. | |
| (Zullo et al., 2005) | To test the efficacy of triple antibiotic therapy plus cMDLf in the eradication of *Helicobacter pylori* infection.<br><br>*Study type:* Prospective, open-label, randomized, multicenter<br><br>*Source:* cMDLf was from Dicofarm (Rome, Italy) | One hundred thirty-three patients (74M/59F) with dyspepsia who were referred by primary care physicians for upper endoscopy were enrolled in the study. All patients underwent endoscopy with biopsies for histology and a rapid urease test. Infection was confirmed if both tests were positive. Patients were randomized to one of two groups: 1) esomeprazole (20 mg 2×/d), clarithromycin (500 mg 2×/d), amoxicillin (1 g 2×/d) (n=68; 39M/29F) for 7dor 2) the antibiotic regimen plus 200 mg cMDLf (2×/d; 400 mg/d total) (n=65) for 7 d. Bacterial eradication was checked 4-6 wk after treatment by using a $^{13}$C-urea breath test. Compliance was defined as consumption of > 90% of the treatment as ascertained by personal interview. | • There was no significant difference in eradication rate of *Helicobacter pylori* infection among the groups. | • All but 3 patients completed the study. Dropouts were due to:<br>Group 1: 2 patients, lost to follow-up<br>Group 2: 1 patient, severe epigastric pain and vomiting<br>• Side effects reported:<br>Group 1: 7 patients; diarrhea, abdominal pain, taste disturbance, pruritis, vomiting<br>Group 2: 6 patients; diarrhea, abdominal pain, glossitis<br>• All side effects were self-limiting after cessation of therapy, and compliance to the therapies was good. |
| (Di Mario et al., 2006) | To test the efficacy of triple antibiotic therapy after administration of cMDLf in the eradication of *Helicobacter pylori* infection.<br><br>*Study type:* Prospective, open-label, randomized, multicenter<br><br>*Source:* cMDLf was from Dicofarm (Rome, Italy) | Four hundred and two *Helicobacter pylori*-positive patients (mean age 52.4 yr; range 19-84 yr) were randomized into one of three groups: 1) esomeprazole (20 mg 2×/d), clarithromycin (500 mg 2×/d), and tinidazole (500 mg 2×/d) (n=136) for 7d, 2) cMDLf (200 mg 2×/d; 400 mg total/d) (n=132) for 7d, followed by the triple antibiotic therapy regimen for 7d, and 3) concurrent treatment with both the triple antibiotic therapy plus cMDLf (200 mg 2×/d; 400 mg total/d) (n=134) for 7d. Infection was assessed at baseline by histology and the $^{13}$C-urea breath test or histology and the *H. pylori* stool antigen-test. | • The eradication rate was significantly higher in group 3 (concurrent administration of cMDLf with triple antibiotic therapy), versus groups 1 and 2 ($p = 0.01$, intent-to-treat analysis; $P = 0.001$, per-protocol analysis). | • Of the 402 patients enrolled, 389 were fully compliant with the therapy, defined as > 95% of study drugs taken.<br>• Major side effects leading to treatment discontinuation by 6 patients included diarrhea and rush (? Possible spelling error in paper) (two patients/group)<br>• One patient in group 2 died because of a street incident.<br>• Six patients were lost to follow up (3 in group 1, 2 in group 2 and one in group 3). |

**Table 19. Human Clinical Studies of cMDLf– Adults**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| (Konishi et al., 2006) | To evaluate the effect of cMDLf on lipid peroxidation, hepatic inflammation and iron metabolism in patients with chronic hepatitis C.<br><br>*Study design:* Randomized, controlled<br><br>*Source:* cMDLf was from Morinaga Milk Industry, Japan | Ninety Japanese patients having chronic hepatitis C (58M/32F) were enrolled. Patients had high serum HCV RNA (> 100 kIU/mL), HCV genotype 1b, consumed < 20 g alcohol/day, and did not have other causes of liver dysfunction. Of the 90 chronic hepatitis C patients, 47 (31M/16F) received 3.6 g cMDLf/d for 8 wk. The remaining 43 chronic hepatitis C patients (27M/16F) did not receive cMDLf and were the control group. Thirty-eight HCV-negative healthy volunteers (15M/23F) served as additional controls. Blood samples were collected for analysis. | • By 8 wk, the cMDLf-treated group had significantly reduced ($p<0.05$) serum alanine aminotransferase (ALT) levels, versus baseline levels at 0 wk. The authors considered this to be an indicator of reduced levels of lipid peroxidation in the body.<br>• Plasma levels of 8-isoprostane were significantly reduced ($p<0.05$), versus baseline levels, by 8 wk in the cMDLf-treated group, but not in the control group. The authors report that 8-isoprostane is a marker of lipid peroxidation. | • None |
| (Paesano et al., 2006) | To study the effect of oral supplementation of cMDLf on iron parameters in pregnant women.<br><br>*Study design:* Not specified<br><br>*Source:* cMDLf was Lf100 from Dicofarm, Rome, Italy; 30% iron saturation | Three hundred pregnant women were included in the study. Women were offered treatment with oral iron supplementation or cMDLf; the control group was composed of women who refused treatment of any kind. Of the 259 women who participated, 54 refused oral iron supplementation (control group), 98 were given 520 mg ferrous sulfate once/day, and 107 were given 100 mg cMDLf twice/day (200 mg cMDLf total/day). Treatment was for 30 d. 520 mg ferrous sulfate delivered 156 mg elemental iron; 200 mg cMDLf delivered 8.8 mg ferric ions. Blood samples were taken and analyzed for hemoglobin and total serum iron at 0 and 30 d.<br><br>*Note:* Inclusion and exclusion criteria were not defined; nor was a detailed analysis plan provided (*e.g.*, intent to treat, etc.) | • The delta mean value was increased for hemoglobin levels in the ferrous sulfate group, versus the control group, at 30 d ($p<0.01$). The increase in delta mean value for hemoglobin levels in the cMDLf group, versus the control group, at 30dwas considered non-significant by the authors ($p=0.02$).<br>• The delta mean value was increased for serum iron levels in both the ferrous sulfate group and the cMDLf group, versus the control group, at 30 d($p<0.01$ for both).<br>• Additionally, the increase in delta mean value for total serum iron was significantly increased in the cMDLf group, versus the ferrous sulfate group, at 30 d ($p<0.01$). | • Thirty-one subjects were excluded or lost to analysis (reason not specified), 4 moved or were lost to follow-up after 30dof treatment, 3 had miscarriages, 3 were excluded for other reasons (not specified).<br>• The authors report that, of the 98 women receiving ferrous sulfate, 95% had stomach pain, cramps and constipation; 2% had at least one episode of diarrhea. Authors report no side effects in the 107 women taking cMDLf. |

**Table 19. Human Clinical Studies of cMDLf– Adults**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| (Ueno et al., 2006) | To evaluate the virologic response to cMDLf in patients having chronic hepatitis C.<br><br>*Study type*: Randomized, double-blind, placebo-controlled, phase III<br><br>*Source*: Morinaga Milk Industries, Tokyo, Japan | Patients 20-74 yr-old who met a detailed criteria indicating established hepatitis C virus (HCV) infection with adequate bone marrow and renal function were assigned randomly to one of two treatment groups in equal proportions using permutation blocks stratified by centers. One hundred ninety-nine patients were enrolled; one refused to participate. Treatments consisting of either 1) cMDLf at a dose of 1.8 g cMDLf/d (as 450 mg/tablets) (n=97) or 2) placebo (n=101) were administered orally twice daily for 12 wk. Serum HCV RNA level and serum alanine aminotransferase (ALT) were measured before treatment, at 4, 8, and 12 wk during treatment, and at 4 wk after treatment. IL-18 and lymphocyte phenotypes were also measured. | • cMDLf treatment did not significantly affect the virologic or biochemical response rates, or significantly alter serum IL-18 levels or CD4+, CD8+, CD16+ and CD56+ peripheral blood lymphocyte populations. | • Three participants in the cMDLf group withdrew due to reasons besides adverse events (not specified).<br>• cMDLf was well tolerated and no serious complications occurred during treatment.<br>• Minor adverse events occurred with similar frequency across both groups and included: neutropenia, γ-GTP elevation and hyperglycemia. |
| (Tursi et al., 2007) | To investigate the efficacy of cMDLf in combination with quadruple antibiotic therapy in *Helicobacter pylori* infected patients who experienced failure of a first antibiotic therapy.<br><br>*Study type:* Prospective, randomized<br><br>*Source:* cMDLf was Elleffe 100® from Dicofarm S.p.A., Rome, Italy | Seventy patients with persistent *Helicobacter pylori* infection after failure of a first standard treatment schedule were enrolled in the study. Infection was confirmed by gastroscopy and biopsy. Patients were randomized into one of two groups: 1) ranitidine bismuth citrate (400 mg 2×/d), esomeprazole (40 mg/d), amoxicillin (1 g, 3×/d), tinidazole (500 mg 2×/d) (n=35) and 2) the regimen listed in 1), plus cMDLf (200 mg 2×/d; 400 mg total/d) (n=35). [**Note:* The length of treatment was not specified.] Endoscopy was carried out at one month post-therapy in those patients "for whom the examination was clinically relevant." In these patients, *H. pylori* presence was ascertained by the rapid urease test and by Giemsa stain. Remaining patients were checked by the $^{13}$C-urea breath test. | • Eradication of *Helicobacter pylori* infection was obtained in 88.57% of patients in group 1 and 94.28% of group 2 patients, based on intent-to-treat analysis. The difference between groups was not statistically significant. | • One patient in group 1 experienced severe diarrhea, vomiting and abdominal pain such that treatment was discontinued.<br>• Nine other patients in group 1 experienced mild side effects that did not require discontinuation of treatment: self-limiting diarrhea, candidosis, abdominal pain, black feces, and nausea.<br>• In group 2, six patients experienced mild or slight side effects that did not require discontinuation of treatment: self-limiting diarrhea, abdominal pain, black feces, nausea, and gastric fullness. |

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| Table 19. Human Clinical Studies of cMDLf– Adults | | | | |
|---|---|---|---|---|
| **Reference** | **Objective/Test Article** | **Study Design** | **Outcome** | **Safety-Related Observations Reported** |
| (Koikawa et al., 2008) | To determine whether cMDLf supplementation would improve or prevent anemia in female long distance runners at high risk of iron-deficiency anemia.<br><br>*Study type:* Double-blind, randomized<br><br>*Source:* cMDLf was from Morinaga Milk Industry, Tokyo; 17% iron saturation, 0.23 mg Fe/g protein | Sixteen female long distance runners approximately 20 yr-old were randomized to one of two groups: 1) 1.8 g cMDLf + 6 mg Fe (as ferric pyrophosphate)/day (n=8) and 2) 6 mg Fe (as ferric pyrophosphate)/day (n=8). cMDLf and Fe were consumed as tablets; the cMDLf tablets contained 0.45 g cMDLf + 5 mg ferric pyrophosphate (1.5 mg as Fe) with 1.05 g carbohydrate; iron tablets contained 5 mg of ferric pyrophosphate (1.5 mg as Fe) plus 1.5 g carbohydrate/tablet. Four tablets were taken daily in each group for 8 wk. Additionally, all subjects were given 30 g of hydrolyzed milk protein in 700 mL water/day. | • cMDLf-supplemented runners did not have the significant decrease in serum iron and ferritin levels, as was observed in the control group.<br>• Compared with the control group, cMDLf-supplemented runners had significantly increased RBC count ($p<0.01$) by 8 wk.<br>• Compared with baseline levels, at 8 wk, the cMDLf-supplemented runners had significantly elevated MCV ($p<0.01$) and MCH ($p<0.05$) values, although final values did not differ significantly from those observed in the control group.<br>• Blood lactate levels after a 3000 m run were significantly lower in the cMDLf group versus the control group at 8 wk ($p<0.05$). Additionally, the increase in lactic acid at 8 wk versus 0 wk was less for the cMDLf group (non-significant) versus the increase observed for the control group, which was statistically significant ($p<0.01$). | • cMDLf supplementation preserved iron status of female long distance runners at-risk of iron-deficiency anemia. |

**Table 19. Human Clinical Studies of cMDLf– Adults**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| (Kondo et al., 2008) [Abstract only available] | To study the effects of oral cMDLf administration in periodontitis patients.<br>*Study design:* Randomized, double-blind, placebo-controlled<br>*Source:* cMDLf was from Morinaga Milk Industries, Japan | Eighteen patients with chronic periodontitis were randomized to one of two groups: 1) cMDLf tablets (n=8); 1.8 g cMDLf/d, information provided by Morinaga) or 2) placebo tablets (n=10) for three mo. Two tablets were administered orally three times a day. Clinical disease indications, total bacterial content (qPCR) of subgingival plaque and saliva and levels of human and cMDLf (ELISA) in the gingival crevicular fluid and saliva were measured. Endotoxin levels in gingival crevicular fluid and saliva were also determined using a Limulus test. | • At 1 wk, *Porphyromonas intermedia* was significantly reduced in the subgingival plaque in the cMDLf group, versus control ($p<0.05$).<br>• A significant reduction in the total number of bacteria was observed in the subgingival plaque after 1 mo in the treatment group, versus control ($p<0.01$).<br>• *Porphyromonas gingivalis* was significantly reduced at 1 and 3 mo in the treatment group, versus control ($p<0.05$).<br>• Levels of cMDLf, but not human cMDLf, were significantly increased in the gingival crevicular fluid and saliva in the treatment group, versus control, at 1 wk and 1 mo ($p<0.001$ for both measurements, at each of the two time points).<br>• Clinical measurements and saliva levels of total bacteria were unchanged by the cMDLf treatment.<br>• There was no change in the levels of endotoxin in the gingival crevicular fluid and saliva of the cMDLf group, versus controls. | • None |

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**Table 19. Human Clinical Studies of cMDLf– Adults**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| (Mulder et al., 2008) | To investigate the immune modulation and antioxidant activity of an oral cMDLf supplement in human males.<br><br>*Study design*: Intraindividual repeated measure; nonblinded<br><br>*Source*: Not stated | Eight healthy males aged 30-55 yr consumed one placebo capsule daily for 7 d, then 100 mg cMDLf/day for 7d, followed by 200 mg cMDLf/day for 7 d. Immune cell populations and function, cytokines and serum antioxidant capacity were measured before and after each supplementation. Medications and supplement use was prohibited 14 prior to and throughout the study. | • T cells were activated during the 200 mg cMDLf/d phase of the study. Statistically significant increases in total (CD3+), helper (CD4+), and cytotoxic (CD8+) T cells were observed on d 16 and 21 ($p<0.001$ for all), versusd0 andd7.<br>• The hydrophilic, but not lipophilic, antioxidant capacity of serum was statistically significantly increased on d 16 and 21 (200 mg cMDLf/d), versusd0 ($p<0.05$). | • None |
| (Bharadwaj et al., 2009) | To measure the effect of milk ribonuclease-enriched cMDLf on bone turnover markers in postmenopausal women.<br><br>*Study design:* Randomized, placebo-controlled<br><br>*Source:* Ribonuclease (angiogenin)-enriched cMDLf was either co-isolated from milk (1:1 ratio, w/w) or separately admixed to this ratio. [Assume purity of cMDLf is ≤ 50%.] | Thirty-five healthy, ambulatory postmenopausal women, 45- to 60-yr-old, with no menses for at least 12 mo were randomized to one of two groups: 1) 250 mg ribonuclease-enriched cMDLf/day (n=20; estimate 125 mg cMDLf/d) or 2) control capsules (n=15) for 180 d. Each group also received an oral supplement containing 100% of the RDA for calcium. Blood and urine samples were collected on d 0, 15, 30, 60, 90, and 180. Analyses were completed on 19 subjects in the cMDLf group and 12 subjects in the control group. | • Bone resorption markers (serum N-telopeptides, $NT_x$, and urine deoxypyridinoline crosslinks, Dpd) were significantly decreased ($p<0.001$ and 0.01, respectively) and bone formation markers (serum bone-specific alkaline phosphatase, BAP, and serum osteocalcin, OC) were significantly increased ($p<0.001$ for both) in the treatment group versus baseline levels. | • None |

**Table 19. Human Clinical Studies of cMDLf– Adults**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| (Kozu et al., 2009) | To determine whether oral cMDLf could inhibit the growth of adenomatous colorectal polyps in human patients.<br><br>*Study design*: Randomized, double-blind, controlled<br><br>*Source*: cMDLf was from Morinaga Milk Industry Co. Ltd.; 10-20% iron-saturated. | Two hundred fifteen patients underwent scheduled colonoscopic examinations and were qualified for enrollment based on results and prior medical history. One hundred eight patients aged 40-75 yr and having ≤ 5 mm-diameter polyps showing a pit pattern III (Kudo's classification) were enrolled in the study. Subjects were randomized and assigned to one of three treatment groups: 1) placebo (n=35), 2) 1.5 g cMDLf (cMDLf)/d (n=37), and 3) 3.0 g cMDLf/d (n=34) for 12 mo. Tablets contained 0, 250, or 500 mg cMDLf/tablet. Subjects took six tablets daily. Intake of products containing cMDLf was prohibited during the study; however, no meals were recorded. Peripheral blood samples were collected at 0, 3, 6, 9, and 12 mo and analyzed for lymphocyte subsets and natural killer (nK) cell activity. Colon polyps were removed at the end of the study and histologically evaluated. | • Polyp growth was significantly inhibited by 3.0 g cMDLf/d in subjects aged ≤ 63 yr-old (n=16) (p=0.006) and in females taking 3.0 g cMDLf/d (n=5) (p=0.019), versus placebo.<br>• Serum levels of human cMDLf were significantly increased in the 3.0 g cMDLf/d group, versus control (p<0.001).<br>• Natural killer cell activity was significantly increased in the 1.5 g cMDLf/d group, versus control (p=0.048). This parameter was also increased in the 3.0 g cMDLf/d group but was not statistically significant versus control (p= 0.058).<br>• Higher serum levels of human cMDLf correlated with reduced invasion of polymorphonuclear leukocytes into the stroma surrounding the target polyps (p=0.021). | •Two subjects were excluded from the trial: one participant assigned to placebo did not have a target polyp; another assigned to the 3.0g cMDLf/d group was found to have used statins.<br>•Two participants from the placebo group withdrew from the study (reason not stated).<br>• Two subjects were excluded from the full analysis set due to use of non-steroidal anti-inflammatory drugs.<br>• In the 3.0 g cMDLf/d group, lung metastases from colorectal cancer were observed in one patient and liver metastases were observed in another patient. Both subjects had a history of colon cancer.<br>• One subject in the 1.5 g cMDLf/d group had a mild increase in alkaline phosphatase levels, and a subject in the 3.0 g cMDLf/d group had a moderate increase in total bilirubin levels during the trial, after an initial mild increase upon commencement of the trial. Both biochemical parameters returned to normal levels at the end of the study.<br>• No other serious adverse events occurred. |

**Table 19. Human Clinical Studies of cMDLf– Adults**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| (Nappi et al., 2009) | To study the effect of cMDLf on iron status and tolerability in pregnant women with iron deficiency anemia.<br><br>*Study type:* Prospective, randomized, controlled, double-blind<br><br>*Source:* cMDLf was Elleffe 100®; Dicofarm, Rome, Italy | 139 pregnant women with iron deficiency anemia who met the following inclusion criteria were evaluated: physiological course of pregnancy, singleton pregnancy, gestational age > 12 wk and < 36 wk, hemoglobin (Hb) values < 11 mg/dL, serum iron (Fe) < 30 µg/dL, serum ferritin < 12 µg/dL and total iron binding capacity (TIBC) > 450 µg/dL. Exclusion criteria included: gestational or persistent disease and fetal abnormalities. 100 women were enrolled and randomized into two groups: 1) 100 mg cMDLf/d (n=50) and 2) 520 mg ferrous sulfate (100 mg diferric Fe) (n=50) for 30 d. Hb, serum ferritin, serum Fe and TIBC were measured at 0 and 30 d. | • Both cMDLf and ferric sulfate supplementation caused a significant increase in Hb, serum ferritin and serum iron at 30 d, versus baseline levels ($p<0.01$).<br>• cMDLf supplementation was as effective as ferric sulfate on the above parameters, based on the magnitude of improvement observed for each measurement. However, the cMDLf group reported fewer side effects versus the ferric sulfate group. | • One patient in the cMDLf group was excluded due to miscarriage.<br>• Two patients in the ferrous sulfate group discontinued treatment due to severe gastrointestinal symptoms.<br>• Median scores of abdominal pain and constipation were significantly higher in the ferrous sulfate group versus the cMDLf group.<br>• cMDLf was well tolerated. |
| (Paesano et al., 2009)<br><br>(Data reported within a review paper) | Data on pregnant women supplemented with cMDLf or ferrous sulfate were included within the review.<br><br>*Study type:* Not specified<br><br>*Source:* Unknown | *Note:* No details were included regarding the inclusion or exclusion criteria, randomization scheme, treatments (besides doses), or statistical plan, etc. The study description within the review mentions the pregnant women are either iron deficient or have iron deficiency anemia, but the criteria which were used to establish this were not reported.<br><br>One hundred forty-three pregnant women were offered iron supplementation. The 33 who refused any treatment were designated as a control group. Sixty women were given cMDLf (dose not specified, but previous study administered 200 mg total cMDLf/day, in two divided doses), and fifty women received ferrous sulfate (dose not stated). Treatment was for 30dand hematological and iron-related parameters were measured at 0 and 30 d. | • Hemoglobin levels were significantly increased in both cMDLf and ferrous sulfate groups ($p<0.0001$ for both), versus baseline levels.<br>• Serum ferritin, total serum iron, and total red blood cells were all significantly increased in the cMDLf group ($p<0.0001$ for all), but not the ferrous sulfate group, versus baseline levels.<br>• Hemoglobin, total serum iron and serum ferritin all declined in the control group, versus baseline levels ($p=0.0042$, 0.0017 and 0.0066, respectively). | • None |

0001115

**Table 19. Human Clinical Studies of cMDLf– Adults**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| (Bharadwaj et al., 2010) | To measure the effect of milk ribonuclease-enriched cMDLf on inflammatory responses in postmenopausal women.<br><br>[Same study as reported on in Bharadwaj et al. 2009?]<br><br>*Study design:* Randomized, placebo-controlled<br><br>*Source:* Ribonuclease (angiogenin)-enriched cMDLf was either co-isolated from milk (1:1 ratio, w/w) or separately admixed to this ratio. [Assume purity of cMDLf is ≤ 50%.] | Thirty-eight healthy postmenopausal women, aged 45-60 yr, with no menses for at least 12 mo were enrolled; three were excluded based on history of treatment for bone health or hypothyroidism. Women were randomized to one of two groups: 1) 250 mg ribonuclease-enriched cMDLf/day (n=20; estimate 125 mg cMDLf/d) or 2) control capsules (n=15) for 180 d. Each group also received an oral supplement containing 100% of the RDA for calcium. Blood samples were collected on d 0, 30, 90 and 180. Cytokine levels were measured. | • IFN-γ levels were reduced (no statistics) at 30, 90 and 180 d in the treatment group, versus placebo.<br>• In general, TNF-α, IL-6, and CRP levels were decreased in both groups at 30 and 90 d, but the magnitude of the decrease was increased in the treatment group, versus placebo. [It should be noted that the initial levels of TNF-α were greater in the treatment group (p=0.07) versus control group.]<br>• IL-10 levels were increased in the treatment group at all time points, relative to the control group.<br>• A small decrease in TGF-β was noted in the treatment group at all time points but it is not known if this is statistically significant or clinically meaningful. The authors report that TGF-β levels vary widely in postmenopausal women.<br>• Effects of treatment on IL-1β, IL-12, and RANKL were either equivocal or not consistent over time. | • There were three drop-outs from the control group before day 15 and one subject from the treatment group was dropped due to non-compliance.<br>• All subjects had > 95% compliance with supplements.<br>• No adverse events were reported during the 6 mo study or 3 mo follow-up period. |

| Table 19. Human Clinical Studies of cMDLf– Adults | | | | |
|---|---|---|---|---|
| **Reference** | **Objective/Test Article** | **Study Design** | **Outcome** | **Safety-Related Observations Reported** |
| (Ishikado et al., 2010) | To study the effect of orally administered cMDLf in subjects with periodontal disease.<br><br>*Study design:* Open intervention<br><br>*Source:* cMDLf was from Morinaga Milk Industry Co., Ltd., Japan | Fourteen volunteers (11M/3F) aged 37-59 having periodontal disease were given a total of 180 mg cMDLf/d as tablets (4/d) formulated as liposomes with soy phosphatidylcholine for 4 wk. Compliance was assessed via pill count and intake record. Probing depth, bleeding on probing, gingival crevicular fluid volume and levels of cytokines and monocyte chemoattractant protein-1 (MCP-1) in gingival crevicular fluid was evaluated over time, and blood samples were taken. | • Levels of MCP-1 in gingival crevicular fluid were significantly decreased at 2 ($p<0.01$) and 4 wk ($p<0.001$), versus wk 0. TNF-α and IL-1β levels were not affected.<br>• At 4 wk, TNF-α and IL-6 ($p<0.05$) and IL-1β and MCP-1 ($p<0.01$) levels in culture supernatants of *Porphyromonas gingivalis* LPS-stimulated peripheral blood monocytes from subjects were significantly decreased, versus 0 wk. No effect of treatment on mRNA expression of Toll-like receptor-2 (TLR2) or -4 (TLR4) was observed. | • Two subjects were dropped from the study due to lack of compliance and extenuating circumstances.<br>• No clinically relevant effects on total protein, aspartate aminotransferase (AST), alanine transaminase (ALT), total cholesterol, high density lipoprotein (HDL)-cholesterol, low density lipoprotein (LDL)-cholesterol, triglycerides, alkaline phosphatase, albumin, A/G ratio, gamma-glutamyltranspeptidase (γ-GT), amylase, urea, nitrogen, ureic acid and creatine, glucose, HbA1c, eruthrocytes, hemoglobin, hematocrit, leukocytes, platelets, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), or mean corpuscular hemoglobin concentration (MCHC) were found. Some significant changes were observed in total protein, A/G radio, ureic acid, HbA1c, MCV, MCHC and monocyte count but all variations were within established normal variation ranges. |

**Table 19. Human Clinical Studies of cMDLf– Adults**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| (Ono et al., 2010) | To determine whether enteric-coated cMDLf would improve visceral fat-type obesity.<br><br>*Study type*: Double-blind, placebo-controlled, randomized<br><br>*Source*: Not stated. | Thirty healthy Japanese men and women over 20 yr-old, with a BMI > 25 kg/m$^2$ and a visceral fat area > 100 cm$^2$ volunteered for the study. Two subjects did not meet inclusion criteria; the remaining 28 were randomized to one of two groups: 1) control (lactose tablets; $n$=14) or 2) enteric-coated cMDLf tablets (300 mg cMDLf/d, taken as 3×100 mg cMDLf/tablets; $n$=14). After a 2 wk run-in period, subjects received three enteric-coated tablets/day for 8 wk. Energy and fat intake were not limited but authors state that supplemental foods or medications known to influence lipid or carbohydrate metabolism were prohibited. Subjects were instructed to maintain their usual dietary intake and physical activity. Subjects fasted overnight for visits at 4 wk intervals at which time anthropometric, circulatory, biochemical and hematological measurements were taken. Interviews were carried out at -2, 0, 4, and 8 wk and computed tomography (CT) was performed at 0 and 8 wk to measure abdominal fat area. One subject was discontinued from each group due to job relocation or work pressure. | • Subjects in the cMDLf group had a statistically significant reduction in weight and BMI at wk 4 ($p<0.05$ for both) and wk 8 ($p<0.01$ for both), versus baseline measurements, and a statistically significant reduction in waist circumference and hip circumference at wk 8 ($p<0.01$ and $P<0.05$, respectively), versus baseline measurements. Similar effects were not observed in the control group.<br>• Subjects taking cMDLf experienced a statistically significant decrease in visceral and total fat area at 8 wk ($p<0.01$ for both), versus baseline measurements. Similar effects were not observed for the control group. | • No effect of cMDLf was observed for systolic or diastolic blood pressure, or pulse rate, versus control.<br>• cMDLf did not affect blood lipids, including total cholesterol, HDL, LDL, triacylglycerides, total lipid, non-HDL cholesterol, and non-esterified fatty acids (nEFA).<br>• No adverse events were observed with regard to safety parameters. |
| (Paesano et al., 2010)<br><br>0001178<br><br>*Note: Two separate clinical trials are reported on.* | To study the effect of orally supplemented cMDLf on iron parameters in pregnant and non-pregnant women. | Subjects for both studies were enrolled when at least one of the four following hematological parameters was deemed indicative of iron deficiency by the study authors: red blood cell count (< 4 × 10$^6$/mL), hemoglobin level (≤ 11 g/dL), total serum iron concentration (≤ 30 mg/dL), and serum ferritin level (≤12 ng/mL). Exclusion criteria included women in their first or second trimester of pregnancy (study #1 only), non-pregnant women (study #1 only), pregnant | *Study #1:*<br>•After 30 d, RBC, hemoglobin, total serum iron, serum ferritin and hematocrit levels increased significantly in the cMDLf group (n=30), versus baseline levels ($p<0.0001$ for all parameters).<br>• Only hemoglobin was increased ($p=0.03$) in the ferrous sulfate group (n=30), versus baseline levels.<br>• None of the measured | *Study #1:*<br>• The authors report that there were no dropouts in the cMDLf group.<br>• Three subjects taking ferrous sulfate dropped out due to side effects.<br>• One subject in the control group dropped out due to severe anemia requiring iron treatment. |

**Table 19. Human Clinical Studies of cMDLf– Adults**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| | *Study type:* Open-label, prospective, randomized<br><br>*Source:* cMDLf was from Lattoglobina, Grunenthal, Italy; iron saturation was ~30%. | women (study #2 only), prior treatment with iron supplements, concomitant diseases, recent blood transfusion and allergy to milk or iron products.<br><br>*Study #1:* Twelve pregnant women refusing iron therapy constituted the control group. Seventy-five pregnant women consenting to iron therapy were randomized into one of two groups: 1) 33 women receiving 520 mg ferrous sulfate once daily with food or 2) 30 women receiving 100 mg cMDLf twice daily before meals (200 mg cMDLf total).Treatments were administered for 30 d. Hematological parameters were measured.<br><br>*Study #2:* Nine women refusing therapy constituted the control group. One hundred eighty-nine women consenting to iron therapy were randomized to one of two groups: 1) 90 subjects receiving 520 mg ferrous sulfate once a day with food or 2) 90 subjects receiving 100 mg cMDLf twice daily (200 mg cMDLf total). Treatments were administered to subsets of subjects for 30, 60, or 90 d. Hematological parameters were measured. | hematological parameters changed significantly by 30din the control group (n=11).<br><br>*Study #2:*<br>• All measured parameters (RBC, hemoglobin, total serum iron, serum ferritin, and hematocrit) were significantly increased ($p<0.0001$ for all) at 30 (n=34), 60 (n=22), and 90 d(n=34) after cMDLf treatment, versus baseline levels/group.<br>• None of the measured hematological parameters changed significantly in the ferrous sulfate groups at any time point, versus baseline levels. | *Study #2:*<br>• The authors report there were no study dropouts in the cMDLf group due to side effects.<br>• Fourteen of ninety subjects taking ferrous sulfate dropped out due to side effects. |
| (Shin et al., 2010)<br><br>0001179 | To test the effects of oral cMDLf and lactoperoxidase on oral malodor and salivary bacteria.<br><br>*Study type*: Randomized, double-blind, placebo-controlled, cross-over | Fifteen healthy volunteers aged 26-54 yr (11M/4F) were randomly assigned to receive either 1) cMDLf (cMDLf; 100 mg cMDLf + 1.8 mg lactoperoxidase + 24.0 mg glucose oxidase/tablet; *n*=8) tablets or 2) placebo tablets (maltitol + cornstarch; *n*=7) daily for 1 day, then underwent a 1 wk washout period before crossing over to the alternate therapy for 1 day. Two tablets were ingested in the morning with a 1 h interval in between doses | • Concentrations of $CH_3SH$ and total volatile sulfur compounds in mouth air were significantly reduced ($p<0.05$ for both) after 10 min treatment with the first tablet of cMDLf, versus control tablets.<br>• Versus baseline levels of $H_2S$, $CH_3SH$, and total volatile sulfur compounds, both cMDLf and control tablets caused an overall decrease (no | • None |

**Table 19. Human Clinical Studies of cMDLf– Adults**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| | *Source*: cMDLf tablets were from Morinaga Milk Industry, Tokyo, Japan | (total of 200 mg cMDLf/d). Tablets were sucked for 10 min and then chewed and swallowed if still remaining. Volatile sulfur compounds and saliva samples were taken just prior to administration and 10, 60 and 120 min after the tablet treatments. | statistics) in these parameters [suggests an inhibitory substance may be present in the control tablets or that a placebo effect exists].<br>• Culture methods and quantitative PCR did not indicate any differences in salivary bacteria populations for the two treatments.<br>• However, terminal restriction fragment length polymorphism analysis (T-RFLP) on the DNA extracted from saliva indicated statistically significant ($p<0.05$ between 0 and 2 h and/or $P<0.05$ between cMDLf and placebo) inhibition of specific bacterial species by the cMDLf tablets at the 2 h time point, versus baseline. [Refer to report for list of organisms.] | |
| (Mueller et al., 2011) | To study the effect of oral supplementation of cMDLf in subjects with mild to moderate facial acne vulgaris.<br>*Study type:* Open-label, prospective, single-center, single-arm (treatment-only)<br>*Source:* cMDLf was supplied as Dermaplus™ chewable tablets (also contained whey milk protein) (manufactured and supplied by Mepha LLC, Aesch, Switzerland; now marketed by Biotan Ltd, Switzerland | Forty-three healthy subjects (18F/21M), aged 17.5 ± 3.8 yr, having dermatologist-confirmed mild to moderate acne vulgaris of the face were enrolled. Subjects with known milk intolerance or allergy were excluded. Subjects took one chewable tablet containing 100 mg cMDLf/tablet twice/day for 8 wk (200 mg cMDLf/d). Based on tablet counts, > 95% of the cMDLf formulation was ingested. Acne lesions were counted at regular intervals during the intervention. | • At 2, 4 and 8 wk treatment, the total count of acne lesions was significantly decreased from baseline levels ($p=0.005$, 0.007 and $p<0.001$, respectively).<br>• The number of inflammatory lesions was significantly reduced only at 2 wk treatment ($p=0.002$).<br>• The number of non-inflammatory lesions was significantly reduced at 4 and 8 wk treatment ($p<0.001$ for both time points). | • Three subjects were lost to follow-up. One subject was excluded due to protocol violation (non-compliance with use of cosmetics).<br>• No serious adverse events and no adverse events leading to discontinuation of test product were noted.<br>• None of the subjects experienced an adverse event considered to be cMDLf-related by the investigator during the study. |

000120

**Table 20. Human Clinical Studies of cMDLf– Children**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| (Okuda et al., 2005) | To study the effect of oral supplementation of cMDLf on *Helicobacter pylori* colonization in humans.<br><br>*Study type:* Randomized, double-blind, placebo-controlled<br><br>*Source:* cMDLf tablets were from Morinaga Milk Industry, Tokyo, Japan | Twenty-five healthy children and 34 healthy adults having *H. pylori* infection either without upper gastrointestinal symptoms or with minimal upper gastrointestinal symptoms who were not being treated were enrolled. *H. pylori* infection was diagnosed when both the $^{13}$C-urea breath test (UBT) and serum- or urine-based enzyme-linked immunosorbent assay (ELISA) were positive. [*Note:* It was not stated what a cutoff for a "positive" value in the UBT test might be.] Subjects who were milk intolerant were excluded. The four treatment groups were: 1) adults consuming two 100 mg cMDLf tablets twice/day (400 mg cMDLf/d) (n=17), 2) adults consuming placebo tablets (n=17), 3) children consuming two 100 mg cMDLf tablets twice/day (400 mg cMDLf/d) (n=14), and 4) children consuming placebo tablets (n=11) for 12 wk. | • The mean UBT values were significantly different at wk 0 between the two child groups ($p<0.01$), which may have introduced a greater tendency for a change in UBT to be observed, versus the adult groups.<br>• After 12 wk supplementation, 10 of 31 (32.3%) subjects in the combined cMDLf groups had a >50% decrease of their UBT value, versus baseline. One of 28 (4%) combined control subjects had a >50% decrease in their UBT value, versus baseline. [*Note:* Baseline UBT values appeared to vary widely across all groups, and it was unclear what constituted a positive result.]<br>• In most of the responders in the cMDLf groups, the UBT values returned to baseline levels by 4 wk after the end of the study. | • None |
| (Egashira et al., 2007) | To demonstrate *in vivo* effects of cMDLf on rotaviral gastroenteritis in children in a day-care setting.<br><br>*Study type*: Open-label, non-randomized<br><br>*Source*: cMDLf was provided as CMDLf Active® dietary food | 298 children under 5-yr-old attending either nursery school or kindergarten (Saga Prefecture, Japan) were enrolled. Milk allergic infants were excluded, and none had chronic illness. Subjects were assigned to one of two groups: 1) 100 mg cMDLf/d for 12 wk (n=136, final analysis) or 2) no cMDLf-containing products (n=98, final analysis). Incidence, duration, and severity of fever, diarrhea, vomiting, and fecal rotaviral antigen | • Frequency ($p=0.0106$) and duration ($p=0.0137$) of vomiting and frequency ($p=0.0446$) and duration ($p=0.0285$) of diarrhea were all statistically significantly decreased in the cMDLf group versus control group.<br>• Number of children with rotaviral gastroenteritis was similar between treatment groups.<br>• There was no significant difference in the duration of fever between the two groups. | • Two children in the control group were hospitalized due to dehydration, compared with none in the cMDLf group. |

0001121

| Table 20. Human Clinical Studies of cMDLf– Children | | | | |
|---|---|---|---|---|
| **Reference** | **Objective/Test Article** | **Study Design** | **Outcome** | **Safety-Related Observations Reported** |
| | supplement tablets (100 mg cMDLf/tablet; Morinaga Milk Industry Co, Ltd., Tokyo, Japan) and CMDLf Yoghurt® (120 g cup yogurt containing 100 mg cMDLf/cup; Morinaga Milk Industry). | were recorded during the study. Subjects were excluded from data analysis if they had diarrheal disease but failed to get the rotavirus antigen test, if the parents did not submit the symptom records, if they left the day-care center during the study, or if they took < 50% of the scheduled dose of cMDLf. 234 subjects were subjected to further analyses. | | |
| (Zuccotti et al., 2007) | To study the immune modulating effects of oral cMDLf supplementation in HIV-infected, antiretroviral-therapy-naïve children.<br><br>*Study design*: Not stated<br><br>*Source*: cMDLf from Dicofarm, Rome, Italy | Eleven HIV-infected, antiretroviral-therapy-naïve children aged 4-17 yr were orally administered 1 g cMDLf every 8 h daily for 4 wk. Blood samples were taken before and after 4 wk of supplementation to measure lymphocyte subsets, intracellular cytokines and phagocytosis and killing by whole-blood leukocytes. | • cMDLf supplementation had no effect on CD4 and CD8 cell counts or HIV plasma viraemia. However, the study was not designed to detect this.<br>• cMDLf supplementation reduced (no statistics) the percent naïve and central memory CD4+ and CD8+ T cells and increased populations of terminally-differentiated lymphocytes at 4 wk, versus baseline.<br>• cMDLf supplementation significantly improved the ability of CD13+ cells to ingest and kill (p=0.01 and 0.009, respectively) labeled *Candida albicans* blastospores at 4 wk, versus baseline.<br>• cMDLf supplementation significantly increased the IL-12:IL-10 ratio in lipopolysaccharide (LPS)-stimulated CD14+ cells (p=0.001) at 4 wk, versus baseline. | |

**Table 20. Human Clinical Studies of cMDLf– Children**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| (Ochoa et al., 2008) | To investigate whether supplementation with cMDLf can prevent diarrhea in children and affect colonization by pathologic microbial species.<br><br>*Study type*: Randomized, double-blind, placebo-controlled<br><br>*Source*: Tatua Nutritionals | Previously weaned children aged 12-36 mo were randomly assigned to receive 1) 0.5 g cMDLf ×2/d (1 g/d × 6 d/wk; average = 0.86 g/d) (n=146) or 2) 0.5 g maltodextrin ×2/d (n=174), on six d/wk for 9 mo.<br><br>Exclusion criteria included children with personal or family history of allergy to cow's milk or infant formula and those who had moderate-to-severe allergic rhinitis or asthma, or milk intolerance.<br><br>Children were evaluated monthly by a physician, and stool samples were collected during diarrheal episodes and on a monthly basis to detect asymptomatic infection. | • There was a statistically significant reduction in the number of positive stool samples for *Giardia lamblia* in the cMDLf group versus the control group ($p<0.05$). | • Height-for-age scores were significantly greater in the cMDLf group versus the control group ($p=0.03$).<br>• There were no adverse events related to cMDLf. |

**Table 21. Human Clinical Studies of cMDLf– Infants**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| (Kawaguchi et al., 1986) | To study the effect of cMDLf-enriched infant formula on premature, low birth weight infants.<br><br>*Study design:* Intervention-only<br><br>*Source:* cMDLf was from Morinaga Milk Industries Co., Ltd., Japan | Sixteen low birth weight infants (> 1500 g weight) who were being bottle fed were enrolled in the study. After reaching stable bottle feeding of approximately 150 mL/kg/d, the infant formula was changed to one that was supplemented with 50 mg cMDLf/100 g powder and the supplemented formula was administered for 2 wk (calculated exposure to cMDLf = 9.75 mg/kg/d). Fecal samples were taken and measures were taken of fecal pH, lysozyme activity, organic acids content, cMDLf level, and intestinal flora. | • During cMDLf supplementation:<br>-- Feces tended to become soft<br>-- Fecal pH declined (second wk)<br>-- Fecal lysozyme activity increased<br>-- Organic acid content of feces increased (especially acetic acid during the second wk)<br>-- The ratio of Bifidobacteria among total bacteria tended to be higher, while the Staphylococcus detection ratio tended to be lower (no statistics provided).<br>• 20-750 µg of cMDLf was detected/g of feces, indicating the relative stability of cMDLf in the low birth weight infant intestinal tract. | • None |
| (Fairweather-Tait et al., 1987) | To measure the effects of cMDLf on iron retention in infants.<br><br>*Study type:* Stable isotope feeding; part of a larger study on cMDLf and fecal flora; group assignment method not reported<br><br>*Source:* cMDLf was from Nestlé Research Department (nestec, Vevey, Switzerland). Study authors prepared their own $^{58}$Fe-labeled cMDLf from this source. | 36 healthy, full-term (36-41 wk) infants (16M/20F) weighing 2060-3800 g who were bottle-fed received either milk-based formula without (40 µg Fe/100 mL; basic formula) or with cMDLf (285 mg cMDLf/100 mL; 86 µg Fe/100 mL, additional iron content was contributed by the added cMDLf; cMDLf formula) on d 1-14. On 7dafter birth, infants were given a single dose of $^{58}$Fe-labeled cMDLf or $^{58}$Fe-labelled ferric chloride plus ascorbic acid. Four groups received the following treatments ond7: 1) those previously fed basic formula who were given 40 µg $^{58}$Fe as $FeCl_3$ (n=8), 2) those previously fed cMDLf-supplemented formula who were given 40 µg $^{58}$Fe as iron-saturated cMDLf (n=8), 3) those previously fed basic formula who were fed 86 µg $^{58}$Fe as | • There were no differences observed across groups for fecal iron concentration or total iron excreted during the 3-day period following dosing of $^{58}$Fe-labeled substrates.<br>• There was no significant difference between the two iron sources, nor did previously fed formula influence iron retention from either $FeCl_3$ or cMDLf. | • Study exclusions included: noncompliance (1), administration of incorrect feed (1), inadvertent pooling of fecal material (3), chronic constipation (2). |

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| Table 21. Human Clinical Studies of cMDLf– Infants | | | | |
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| **Reference** | **Objective/Test Article** | **Study Design** | **Outcome** | **Safety-Related Observations Reported** |
| | | $FeCl_3$ (n=8), and 4) those previously fed cMDLf-supplemented formula who were fed 86 µg $^{58}Fe$ as iron-saturated cMDLf (n=5). All subsequent diapers were collected for 3dafter dosing of labeled Fe. Iron retention was calculated by subtracting fecally excreted $^{58}Fe$ from the administered dose, while adjusting for naturally occurring $^{58}Fe$ and loss due to leftover formula or vomiting. | | |
| (Kawaguchi et al., 1989) | To study the effect of cMDLf-enriched infant formula on premature, low birth weight infants.<br><br>*Study design:* Intervention-only<br><br>*Source:* cMDLf was from Morinaga Milk Industries Co., Ltd., Japan | Nine low birth weight infants (1454-2034 g weight; 29-36 wk gestational age) who were being bottle fed were enrolled in the study. After reaching stable bottle feeding of approximately 150 mL/kg/d and allowing 10 or more d to elapse following the discontinuation of any antibiotic treatments, the infant formula was supplemented with 100 mg cMDLf/100 mL and the supplemented formula was administered for 2 wk (calculated exposure to cMDLf = 150 mg/kg/d). There was an additional 1 wk follow-up period after the study formula was discontinued. Fecal samples were taken and measures were taken of fecal pH, lysozyme activity, organic acids content, cMDLf level, and intestinal flora. | • By wk 2, the composition ratio of *Bifidobacterium* and *Veillonella* culturable species in the fecal flora was increased relative to baseline levels (no statistics). This effect persisted and was still observed 1 wk after cMDLf discontinuation.<br>• There was a transient increase in the composition ratio of *Bacteroidaceae* species at 2 wk, versus baseline levels, but this effect disappeared by 1 wk post-study.<br>• By 2 wk, the composition ratio of *Enterobacteriaceae* and *Clostridium* culturable species had declined, relative to baseline levels. This effect persisted and was still observed 1 wk after cMDLf discontinuation.<br>• 8-9 mg of cMDLf was detected/g of feces, indicating the relative stability of cMDLf in the low birth weight infant intestinal tract.<br>• No other significant effects were noted for fecal pH and organic acids (acetic, lactic acids) content. | |

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**Table 21. Human Clinical Studies of cMDLf– Infants**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| (Chierici et al., 1992) | To study the effect of cMDLf supplementation in infant formula on serum hemoglobin, hematocrit, ferritin, iron and zinc levels in infants.<br><br>*Study type:* Not specified; randomization unclear<br><br>*Source:* cMDLf was from Oleofina, Brussels, Belgium, non-heat-treated, 20% iron-saturated | Healthy, full-term Italian infants were recruited with parental consent (both sexes; 51 parents consented). Infants were assigned (protocol not stated) to one of four groups: 1) breast milk (n=10), 2) control formula (n=13), 3) formula containing 10 mg cMDLf/100 mL (n=14), 4) formula containing 100 mg cMDLf/100 mL (n=14). Iron content of the control formula was 70 µg/100 mL; of the low-cMDLf-supplemented formula was 72.8 µg/100 mL; of the high-cMDLf-supplemented formula was 98 µg/100 mL. Formula was fed for 150dand serum samples were taken at 0, 7, 30, 90 and 150 d. | • There were no statistically significant differences in hemoglobin or hematocrit between the groups at any sampling time.<br>• Byd30, breast-fed infants had significantly lower serum iron levels versus control formula-fed infants (p=0.012) and infants in the high cMDLf group (p=0.041).<br>• Serum ferritin increased in all groups fromd0 tod7, held steady atd30, then declined byd30. Atd150, the high cMDLf group had significantly higher serum ferritin versus the control formula group (p=0.02). (*note*: infants in the high cMDLf group received slightly more iron versus the other two formula groups.) However, by this time all serum ferritin levels had significantly declined versusd0 across all groups.<br>• No statistically significant differences in serum zinc levels were observed between the groups at any sampling time. | • Two breast-fed infants had low hemoglobin levels atd90. |
| (Roberts et al., 1992) | To study the infant fecal flora of infants consuming an adapted formula containing cMDLf.<br><br>*Study design*: Unknown<br><br>*Source*: cMDLf from Oleofina, Brussels, Belgium; 20% iron-saturated. | Healthy full-term infants were recruited and fed one of four diets from birth to at least the end of the 3rd mo of life: 1) breast milk (n=12), 2) Preaptamil adapted formula (Milupa AG, Friedrichsdorf, Germany) (n=14), 3) Preaptamil + 10 mg cMDLf (cMDLf)/100 mL (n=15), 4) Preaptamil + 100 mg cMDLf/100 mL (n=14). Protein content was normalized across the cMDLf- | • 57% of infants in the 100 mg cMDLf/100 mL formula-fed group exhibited a bifidobacteria-dominated spectrum (no statistics) atd90, which was similar to the prevalence of bifidobacteria-dominated flora observed for the breast milk-fed group (50%) at this time point. However, the "bifidus flora" effect did not appear untild90 and was not observed in the 10 mg cMDLf/100 mL formula-fed | |

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| Table 21. Human Clinical Studies of cMDLf– Infants | | | | |
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| **Reference** | **Objective/Test Article** | **Study Design** | **Outcome** | **Safety-Related Observations Reported** |
| | | supplemented formulas by removal of an equivalent amount of whey protein. Formula was fed for 3 mo. Fecal samples were collected at 1 wk, 1 mo, and 3 mo after birth, and cultured for fecal flora content. A "bifidus flora" was defined as a flora where bifidobacteria outnumbered all other measured components of the fecal flora by 1 $\log_{10}$ CFU/g of feces. Fifty-one infants finished the study. *Note*: introduction of outside weaning food was not restricted. | group; therefore, the authors did not consider cMDLf to have provided a beneficial effect of increasing early fecal colonization by bifidobacteria.<br>• The authors commented that the 20% iron-saturation level of the cMDLf utilized may have negated the expected inhibition of the non-bifididobacteria species. | |
| (Lönnerdal and Hernell, 1994)<br><br>0001277 | To study the hematologic effects of iron, selenium, and copper supplementation on infant growth, and iron and copper status.<br><br>*Study type:* Randomized, double-blind<br><br>*Source:* Not specified | Healthy term infants were either exclusively breast-fed or fed milk-based infant formula from 6-wk-of-age to 6-mo-of-age (n=10/group).<br><br>Treatment groups are: A) 4.3 mg/L $FeSO_4$, 5 µg /L selenium, 0.4 mg/L copper; B) 4.4 $FeSO_4$, 15.6 µg /L selenium, 0.4 mg/L copper; C) 1.3 mg/L bovine lactoferrin, 2.5 mg/L $FeSO_4$, 15.6 µg /L selenium, 0.7 mg/L copper; D) 4.7 mg/L $FeSO_4$, 3.9 µg /L selenium, 0.46 mg/l copper; E) 6.9 mg/L $FeSO_4$, 5.0 µg /L selenium, 0.4 mg/L copper; F) breast milk<br><br>Anthropometric measurements and venous blood samples were obtained on entry into the study and at 6-mo-of-age.<br><br>Only comparison between infants that received breast milk and infant formula supplemented with bovine lactoferrin are relevant. | • No significant differences in birth weight or height at 6 weeks or 6 mo of age.<br>• There were no differences among the formula-fed groups for serum albumin, BUN, hemoglobin, ferritin, serum transferrin receptor concentration, zinc,<br>• Although the copper, a2-macroglobulin, selenium, and glutathione peroxidase levels were higher in the breast-fed infants, there were no differences in these parameters among the formula-fed groups. | • No significant differences in birth weight or height at 6-weeks or 6-mo-of-age.<br>• No significant differences in hematological indices; all infants had satisfactory iron status. |

| Table 21. Human Clinical Studies of cMDLf– Infants | | | | |
|---|---|---|---|---|
| **Reference** | **Objective/Test Article** | **Study Design** | **Outcome** | **Safety-Related Observations Reported** |
| (Hernell and Lönnerdal, 2002) | To study the hematologic effects of iron supplementation at various levels and with or without cMDLf or nucleotides in infant formula.<br><br>*Study type:* Not specified<br><br>*Source:* cMDLf was from SMR, Malmö, Sweden; cMDLf was iron-saturated with Fe content of 1.24 mg/g protein; purity was 95% protein, of which 95% was cMDLf | Healthy term infants were initially breast-fed and then allocated to either a breast-fed group (n=16) or various formula-fed groups (n=10-12/group), according to parent choice. Infants in the formula-fed groups were completely weaned from breast milk. The formula groups were as follows: 1) formula containing 1.6 mg Fe/L (n=12; Fe as $FeSO_4$), 2) formula containing 1.8 mg Fe/L, 1.3 mg Fe was provided as cMDLf, 0.5 mg was $FeSO_4$ (n=10; calculated as 1.05 g cMDLf/L), 3) formula containing 2.2 mg Fe/L (Fe as $FeSO_4$) plus 40 mg monophosphate nucleotides/L (n=10), 4) formula containing 4.0 mg Fe/L (Fe as $FeSO_4$) (n=11). Formula was fed for 6 mo. Anthropometric measures and blood samples were taken at 1, 4, and 6 mo. | • At 6 mo, mean body weight and height of the cMDLf-supplemented group was not significantly different from that of the breast-fed, 1.6 mg Fe group, or 4.0 mg Fe group. Weight and height were significantly higher ($p<0.05$) versus that of the 2.2 mg Fe plus nucleotide group.<br>• cMDLf supplementation did not affect serum iron, ferritin, hemoglobin or other hematological parameters at any time point.<br>• cMDLf supplementation did not affect serum zinc, copper, or the fatty acid composition, except docosahexanoic acid (DHA), of the erythrocyte membrane at any time point. All formula groups exhibited significantly lower levels of DHA in the erythrocyte membrane at 4and 6 mo, versus the breast-fed group ($p<0.05$). | • No effect of cMDLf on body weight or height at 6 mo, except versus that of the 2.2 mg Fe plus nucleotide group ($p<0.05$).<br>• No effect of cMDLf on iron parameters at any time point. |

**Table 21. Human Clinical Studies of cMDLf– Infants**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
|---|---|---|---|---|
| (King et al., 2007) | To examine the impact of cMDLf supplementation in infants over one yr.<br><br>*Study type*: Randomized, placebo-controlled, double-blind<br><br>*Source*: cMDLf was provided by DMV International, Delhi, NY. Iron content of cMDLf was 120 µg/g powder. | Healthy infants aged 0-4 wk, born at ≥ 34 wk gestation at ≥ 2000 g weight, and who were strictly bottle-fed were eligible for the study. Infants were randomized to receive powdered Similac with iron formula (3 mg/L elemental iron) either with (850 mg/L) or without (102 mg/L; basal cMDLf present in cow's milk-derived formula) added cMDLf for 12 mo. Weight, length, and head circumference were measured at 1, 2, 4, 6, 9, and 12 mo of age. Incidence and duration of infectious disease endpoints were tracked, including diarrhea, upper respiratory infection, acute otitis media, lower respiratory tract infection; plus, other endpoints were followed such as colic, hemoglobin, hematocrit, mean corpuscular volume, and antibody response to various vaccines.<br><br>Seventy-nine infants were enrolled in the study; 52 completed the full-yr study period. Of the 52 completers, 26 received the cMDLf-supplemented formula and 26 received the control formula. | • CMDLf-supplemented infants had statistically significantly decreased occurrence of lower respiratory tract infections versus the control group ($p<0.05$). | • There was a trend for increased weight over time up to 6 mo in the cMDLf-supplemented group, but it did not reach statistical significance ($p=0.06$). *Note*: Authors state that the added cMDLf slightly increased the total protein content of the treatment formula by ~5%, and this may have influenced the growth and weight change differences between groups.<br>• CMDLf-supplemented infants had a statistically significantly increased hematocrit at 9 mo ($p<0.05$), and a trend toward increased mean corpuscular volume (MCV) at 12 mo ($p=0.06$), versus control.<br>• 13/27 dropouts received cMDLf-supplemented formula. Of the 27 total dropouts, 19 withdrew due to intolerance (10 received cMDLf), withdrawal of consent (n=3), or were lost to follow-up (n=5). |

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**Table 21. Human Clinical Studies of cMDLf– Infants**

| Reference | Objective/Test Article | Study Design | Outcome | Safety-Related Observations Reported |
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| (Manzoni et al., 2009) | To establish whether cMDLf, alone or in combination with *Lactobacillus rhamnosus* GG, reduces the incidence of late-onset sepsis in very low birth weight neonates.<br><br>*Study type*: Prospective, multicenter, double-blind, placebo-controlled, randomized<br><br>*Source*: LF100; Dicofarm SpA, Rome, Italy | Very low birth weight (VLBW) neonates younger than 3 d were enrolled and randomly allocated into one of three groups: 1) 100 mg cMDLf/d (n=153), 2) 100 mg cMDLf/d plus $6 \times 10^9$ CFU *Lactobacillus rhamnosus* GG/d (n=151), or 3) placebo (2 mL of a 5% glucose solution) (n=168). Treatment lasted 6 (birth weight <1000 g) or 4 (birth weight 1001-1500 g) wk, beginning on d 3 of life with one daily dose. All treatments were diluted in prepared milk. Breast-feeding was encouraged, and supplemented with a VLBW formula when necessary (did not contain cMDLf). Adverse events were recorded until 2 d after the end of the study. Liver function was evaluated wkly. | • Infants in the cMDLf group had statistically significantly decreased incidents of total late-onset sepsis (p=0.002); Gram-positive bacteria (p=0.007); and Gram-positive bacteria, including episodes diagnosed with ≥ 1 positive blood culture for coagulase-negative *Staphylococcus* spp. (p=0.002), versus controls.<br>• The reduction in late-onset sepsis was statistically significant for extremely low birth weight infants (p=0.002), neonates weighing ≤ 750 g (p=0.003), and neonates ≤ 27 wk gestational age at birth (p=0.01) in the cMDLf group, versus controls.<br>• Invasive fungal infection was reduced in total incidents (p=0.004) and in extremely low birth weight neonates (p=0.03) in the cMDLf group, versus controls.<br>• Progression rate from colonization to invasive fungal infection was significantly reduced in all neonates for the cMDLf group versus control (p=0.002).<br>• Threshold retinopathy of prematurity requiring surgery was also reduced in the cMDLf group versus control (p=0.02). | • No adverse effects or intolerances to treatment occurred.<br>• One infant in the cMDLf group discontinued treatment but was included in the intent-to-treat analysis.<br>• Nine infants had incomplete data on certain variables but not for analysis of study outcomes.<br>• At age 4 wk, liver enzyme values were within reference ranges but were significantly lower (p value not provided in main article) in both treatment groups versus control.<br>• Incidence of hyperbilirubinemia requiring phototherapy was similar in the three groups.<br>• No infants exhibited signs of hepatotoxicity or cholestasis.<br>• Mortality attributable to sepsis was significantly reduced in the cMDLf group, versus control (p=0.008). |

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# GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR COW'S MILK-DERIVED LACTOFERRIN AS A COMPONENT OF COW'S MILK-BASED INFANT FORMULAS, COW'S MILK PRODUCTS, AND CHEWING GUM

We, the members of the Expert Panel, qualified by scientific training and experience to evaluate the safety of food and food ingredients, have performed a comprehensive and critical review of available information and data on the safety and Generally Recognized As Safe (GRAS) status of the use of cow's milk-derived lactoferrin (cMDLf) in cow's milk-based infant formulas [powdered (100 mg/100 g), liquid concentrates (26 mg/100 ml), and ready-to-feed formulas (13 mg/100 ml)], yogurt (100 mg/100 g), powdered milk (400 mg/100 g), ice cream and sherbets (200 mg/ 100 g), and chewing gum (30 mg/g) is based upon scientific procedures as described under 21 CFR §170.30(b). The data and information are summarized in this GRAS determination document, *Generally Recognized As Safe (GRAS) Determination for Cow's Milk-Derived Lactoferrin as a Component of Cow's Milk-Based Infant Formulas, Cow's Milk Products, and Chewing Gum*, prepared by Spherix Consulting, Inc., for Morinaga Milk Industry Co., Ltd., and that is appended herewith.

Based upon our review of the information and data available, we find that the intake of cMDLf from the intended uses specified has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), Section 201(s). To demonstrate that cMDLf is safe, and GRAS, under the intended conditions of use, the safety of the intake of cMDLf has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

The proposed use of cMDLf to supplement levels of this protein in selected cow's milk-based food products has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. In the United States, cMDLf has been determined safe and GRAS for use as an ingredient in sport and functional foods at concentrations of 100 mg/serving (GRN 77). cMDLf has also been determined safe and GRAS for use as a component of an antimicrobial spray for application to uncooked beef to prevent microbial contamination under GRN 67 and GRN 130.

2. cMDLf has a long and safe history of ingestion. Milk from cows has been consumed by the human population for centuries. Milk protein is a combination of caseins and whey proteins. Caseins account for 79.2% (27 mg/ml milk) of the total milk proteins. The remaining 20.8% (7.1 mg/ml milk) is whey protein. cMDLf, which is a whey protein, accounts for 0.3% (0.1 mg/ml milk) of the total protein or 1.4% of whey protein in milk.

3. cMDLf manufactured for Morinaga Milk Industry Co., Ltd. complies with established food grade specifications and utilizes only food grade raw materials and processing aids. The product consists of 4.2% moisture, 1.3 % ash, and 94.5% protein, of which ≥96% is lactoferrin. The current production facilities comply with either the requirements of International Food Standard Version 5 or ISO 9001:2008 and ISO 14001:2004.

4. Infant formulas with added cMDLf are approved for use and sold in Japan, Taiwan, Pakistan, Indonesia, and China by Morinaga Milk Industry Co., Ltd. In Japan, the formulas have been certified by the Japanese Ministry of Health, Labor, and Welfare as Special Nutritious Foods according to the Nutrition Improvement Law. Morinaga's cMDLf has been listed on the "natural additive list" in Japan since 1989 and was added to the "existing additive list" in Japan in 1995. In Japan, there is no specific restriction for cMDLf because it is considered a natural material. The proposed GRAS level of cMDLf (100 mg/100 g powdered infant formula) to be added to cow-milk based infant formula is similar to the level of cMDLf in currently marketed and consumed Morinaga Milk infant formula products (80 mg/100 g powdered infant formula).

5. Since the release of Morinaga's infant formula products in 1986 and 1989, Morinaga has sold annually approximately 3,200 metric tons (76,800 metric tons total) and 3,500 metric tons (73,500 metric tons total) in Japan for the 0- to 9-month formulation and 9-month to 3-year formulation, respectively. With consumption of these formulas with added cMDLf by over a million infants and toddlers in Japan since 1986, there have been no reported significant health problems, including allergenic reactions or autoimmunity attributable to cMDLf, associated with either of these two products containing cMDLf based on post-marketing surveillance.

6. The mean EDIs of cMDLf from addition to infant formulas are 100.3 mg/day for infants 0 to 4 months and 87.4 mg/day for infants 5 to 11 months. The 90$^{th}$ percentile intakes for these age groups are 145.5 mg/day and 129 mg/day, respectively. After cMDLf addition to infant formulas, the cMDLf exposure to this protein for infants in these age categories is approximately two-times the background exposure from unsupplemented cow-milk based infant formula.

7. The mean and 90th percentile EDIs by the total population of cMDLf from addition to proposed products excluding infant formulas are 142 mg/day and 273 mg/day (2.7 mg/kg/day and 5.8 mg/kg/day, respectively). Similarly, for all other population groups, the exposure to cMDLf from addition to the proposed products increases exposure by two- to three-times background.

8. The EDIs for cMDLf have been concluded to be safe and GRAS based on ADME studies, animal toxicology studies, studies evaluating physiologic effects, and clinical studies in adults, children, and infants. These published studies support the safety of intake of cMDLf at the proposed levels.

9. Digestion and metabolic fate of lactoferrin has been evaluated from studies of both human milk-derived and cow's milk-derived lactoferrin. Lactoferrin from both sources is handled similarly by the body. Lactoferrin that is absorbed from the gastrointestinal tract partitions into the lymph and then appears in the blood. Lactoferrin is rapidly removed from the systemic circulation by distribution into the spleen, liver, and kidneys while the iron portion is transferred to the liver for its transport into the bone marrow. Intact lactoferrin is detected in the feces and urine of infants.

10. While very few patients with cow's milk allergy (CMA) have antibodies to bovine lactoferrin, no evidence exists that cMDLf is a clinically relevant allergen.

11. It has been shown that infants fed with formula containing added cMDLf developed anti-cMDLf antibodies. Exclusively breastfed infants develop anti-human lactoferrin antibodies, and autoimmune adults have antibodies that recognize both human and cMDLf. However, there is no evidence that cMDLf causes, promotes, exacerbates, or resolves autoimmunity in infants or adults.

12. The safety of cMDLf produced by Morinaga Milk Industry Co., Ltd. was evaluated in an acute toxicity study, a four-week oral toxicity study, a thirteen-week oral toxicity

study, a chronic oral toxicity study and genotoxicity assays. cMDLf is not acutely toxic or genotoxic. cMDLf administered by oral intubation to rats for 13 weeks did not result in toxicologically significant treatment-related changes. Thus, under the conditions of this study, the no-observed-adverse-effect level (NOAEL) of cMDLf was estimated to be in excess of 2,000 mg/kg/day. The chronic toxicity study was not available as a full report and therefore was not used to derive a NOAEL.

13. Numerous published studies on the effects of oral ingestion of cMDLf in adults, children, and infants corroborate the GRAS status of cMDLf for its proposed uses. Of the 37 studies in humans, 17 have been conducted using cMDLf supplied by Morinaga Milk Industry. cMDLf is safe and well-tolerated under the conditions of administration in the clinical studies at doses that range in adults and children from 100 mg/day to 3.6 g/day and with durations ranging from one week to one year. Studies carried out in both healthy and health-compromised adults and children reported no treatment related side effects attributed to oral ingestion of cMDLf. Administration of cMDLf at 200 mg/day for up to 90 days in pregnant women was well tolerated (highest dose tested in this target population). In term and preterm infants, exposure to cMDLf from infant formulas has been studied using concentrations ranging from 10 mg/100 mL (0.01%) to 285 mg/100 mL (0.285%), comprising durations ranging from two weeks to one year. Resulting intake of cMDLf is up to 150 mg/kg/day. No treatment related adverse effects have been reported in infants.

Determination of the GRAS status of cMDLf supplied by Morinaga Milk Industry under the intended conditions of use has been made through the deliberations of Dr. Stephen Taylor, Dr. Lloyd Mayer, Dr. A. Wallace Hayes, and Dr. Roger Clemens. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of cMDLf and the potential human exposure to cMDLf resulting from its intended use in milk-derived products and infant formulas and have concluded:

There is no evidence in the available information on cMDLf that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when cMDLf is used at levels that might reasonably be expected from the proposed applications. cMDLf is GRAS for use in foods as proposed by Morinaga Milk Industry Co., Ltd.

Therefore, cMDLf is safe and GRAS at the proposed levels of addition to foods. cMDLf is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

It is our opinion that other experts qualified by training and/or experience to evaluate the safety of food and food ingredients would concur with these conclusions.

Roger A. Clemens, DrPH, CNS, FACN, FIFT
GRAS Expert Panel Member
University of Southern California
School of Pharmacy

Signature: (b) (6)

Date: 6/22/11

A. Wallace Hayes, PhD, DABT, FATS, ERT
GRAS Expert Panel Member
Harvard School of Public Health

Signature: (b) (6)

Date: 6/22/11

Lloyd Mayer, MD
GRAS Expert Panel Member
Mount Sinai School of Medicine

Signature: (b) (6)

Date: 6/22/11

Stephen L. Taylor, MS, PhD
GRAS Expert Panel Member
University of Nebraska

Signature: (b) (6)

Date: 22 June 2011

Claire L. Kruger, PhD, DABT
Scientific Advisor to the Panel
Spherix Consulting, Inc.

Signature: (b) (6)

Date: 6/22/11

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**Ramos-Valle, Moraima**

**From:** Kruger, Claire [ckruger@spherix.com]
**Sent:** Wednesday, February 29, 2012 1:53 PM
**To:** Ramos-Valle, Moraima
**Cc:** Brailer, Kathy
**Subject:** RE: GRAS determination for "milk-derived lactoferrin"
**Attachments:** image001.png



Dear Moraima:

As we discussed, please note below the following edit (in bold) to the GRAS Notification for the document:"GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR COW'S MILK-DERIVED LACTOFERRIN AS A COMPONENT OF COW'S MILK-BASED INFANT FORMULAS, COW'S MILK PRODUCTS, AND CHEWING GUM":

The data and information that serve as the basis for this GRAS determination **will be sent to FDA upon request or** will be available for review and copying at reasonable times at the office of Claire L. Kruger, Ph.D., D.A.B.T., CEO, Spherix, Incorporated, 6430 Rockledge Drive, Westmoreland Bldg, Suite 503, Bethesda, Maryland, 20817, Telephone: 301-897-0611; Facsimile: 301-897-2567; Email: ckruger@spherix.com.

The document already contains the following statement:

Pursuant to the criteria provided in proposed 21 CFR 170.36, Morinaga Milk Industry Co., Ltd. hereby notifies the Food and Drug Administration that the use of cMDLf in foods under the intended conditions of use is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because Morinaga Milk Industry Co., Ltd. has determined that such use is Generally Recognized As Safe through scientific procedures.

Please let me know if this is satisfactory or if you have any additional questions.

(b) (6)

Claire

Claire Kruger, PhD., D.A.B.T.
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SUBMISSION END